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ALTERATION OF VASCULAR ENDOTHELIAL GROWTH FACTOR A (VEGFA) ISOFORM EXPRESSION RESULTS IN ABNORMAL GONADAL FUNCTION

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**ALTERATION OF VASCULAR ENDOTHELIAL GROWTH
FACTOR A (VEGFA) ISOFORM EXPRESSION RESULTS IN
ABNORMAL GONADAL FUNCTION**

By

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A THESIS

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Alteration of VEGFA isoform expression results in abnormal gonadal development

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University of Nebraska, 2010

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In the female, vascular endothelial growth factor A (VEGFA) isoforms regulate follicle development and affect the initial primordial follicle pool. In male rodent testes, they are involved in the development of vasculature and seminiferous cords. The objective of the current study was to evaluate effects of Sertoli and Granulosa cell-specific production of VEGFA isoforms on vascular development and gonadal morphogenesis. We used a DMRT1-cre crossed to a floxed VEGFA mouse to determine potential phenotypes in male Sertoli-cell (VEGFA-DSertG) and female Granulosa-cell (VEGFA-DGranG) specific knockouts. In females, we found smaller ovarian weight, fewer corpus luteums (CLs) with some abnormal CL morphology, and 55% reduction in estrogen in the VEGFA-DGranG compared to controls. The number of days mated until parturition was 12 days longer in VEGFA-DGranG X VEGFA-DSertG pairs versus control females mating with control males. In males, the weight of body, testes and epididymides in VEGFA-DSertG mice were significantly reduced than those from the controls. The morphology of epididymis and prostate appeared to be different with less tubules and branching, respectively compared to controls. Serum testosterone levels in VEGFA-DSertG tended to be higher than those in controls. *Bcl-2* mRNA and *Bcl-2/Bax*

in VEGFA-DSertG testis tended to be higher than that in controls. *Sin3a* mRNA within testis was higher in those three VEGFA-DSertG males with high serum testosterone concentration than controls. Therefore, we conclude that lack of VEGFA in Granulosa and Sertoli cells alters reproductive organ morphogenesis, reduces gonadal and male accessory gland organ weights, alters steroidogenesis and creates sub-fertility in mice.

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Chapter 1

Literature Review

Introduction

Over the past decades, fertility has sharply declined and the proportion of couples who need infertility treatment has increased tremendously (Leridon *et al.*, 2008). About one-half of infertility cases are due to male factors such as cryptorchidism, oligozoospermia and the other one-half are attributed to female such as uterine and pelvic abnormalities, secondary infertility, polycystic ovarian syndrome. Currently increased human infertility suggests that significant reproductive health problems exist (Skakkebaek *et al.*, 2005).

VEGFA is also present during testis morphogenesis and expressed by Sertoli cells which play an important role in the testis formation and function. The incidence of male reproductive disorders including impaired spermatogenesis, undescended testis, hypospadia and testicular cancer has been increasing during recent decades. These reproductive disorders could be called testicular dysgenesis syndrome (TDS) (Funke *et al.*, 2010). One potential cause of TDS may be due to disruptions in embryonic gonadal development. Our laboratory has demonstrated that inhibition of VEGFA angiogenic isoforms arrested both testis cord formation and vascular development in mice (Bott *et al.*, 2006). Excess of VEGFA angiogenic isoform resulted in less organized and poorer seminiferous cords in rat testis (Baltes-Breitwisch *et al.*, 2010). VEGFA signal transduction inhibitors reduced the endothelial cell migration during testis formation in

mice (Bott *et al.*, 2010). Furthermore, overexpression of VEGFA in transgenic mice results in infertility (Korpelainen *et al.* 1998).

Vascular Endothelial Growth Factor A (VEGFA) is paracrine growth factor responsible for blood vessel development as well as endothelial cell migration. VEGFA expression and production within the ovary are critical for normal reproductive function. In the female, VEGFA plays an important role in the cyclic growth of ovarian follicles and in the development and maintenance of corpus luteum (CL) by mediating ovarian angiogenesis. Defects in angiogenesis may cause some disorders such as anovulation, pregnancy loss, ovarian hyperstimulation syndrome, and ovarian neoplasms (Geva and Jaffe, 2000). Previous studies in our laboratory demonstrated that VEGFA is involved in primordial follicle activation, maturation and survival (McFee *et al.*, 2009; Artac *et al.*, 2009).

The objective of my research is to evaluate the effect of granulosa and Sertoli cell-specific production of VEGFA isoforms on vascular development and gonadal morphogenesis.

Testis morphogenesis

There are at least five morphological stages of testis development: (1) development of a genital ridge, (2) formation of an indifferent or bipotential gonad, (3) sex determination, (4) induction of testicular cords in the testis, and (5) development of a functional testis (Fig. 1) (Cupp and Skinner 2005).

Development of a genital ridge

Development of a genital ridge occurs in both male and female. Formation of the genital ridge and urogenital system from the intermediate mesoderm starts at 9-9.5 days post coitus (dpc) in the mouse. In male, Woffian duct formation is driven by lateral mesoderm and develops from the mesonephric duct (Grobstein, 1953). Several genes such as *Lhx1* (LIM homeobox 1), Wilms' tumor 1 (*Wt1*) and *Emx2* are expressed in the urogenital ridge by 9.5 dpc in the mouse and contribute to the development of the indifferent gonads (Shawlot *et al.*, 1995; Kreidberg *et al.*, 1993; Miyamoto *et al.*, 1997). At approximately 10.5 dpc, the gonad forms as an outgrowth of the genital ridge in both male and female (Brennan *et al.*, 2002). Then, the coelomic epithelium of the urogenital ridges will develop to the indifferent gonads (Cui *et al.*, 2004).

Formation of an indifferent gonad

The second stage of testis morphogenesis is the development of indifferent gonad. In the mouse, the indifferent gonad becomes visible at 10 dpc but still cannot be identified as a testis or ovary (Swain and Lovell-Badge, 1999). The indifferent gonad can differentiate to testis or ovary depending on the transcription factors expressed in the differentiating somatic cells of the coelomic epithelium (Cupp and Skinner, 2005). In XY gonads, primordial germ cells (PGCs) migrate long distances from the yolk sac to the prospective gonads (Aman and Piotrowski, 2010). The migration of PGCs in the mouse is induced at the interface between extraembryonic and embryonic tissues. Bone morphogenetic protein (BMP) 4 and 8 signaling from the extraembryonic ectoderm is

required for this induction (Kunwar *et al.*, 2006). The migration of PGCs is from the gut to somatic gonadal mesoderm to colonize the gonads (Bendel-Stenzel *et al.* 1998, Molyneaux and Wylie, 2004). Kit ligand (KL) plays a critical role in regulation of PGC survival by preventing PGC apoptosis. Kit Ligand also promotes PGC migration due to its function as an adhesion factor (De and Pesce, 1994; De *et al.*, 2009).

Sex Determination

The third stage of testis development is sex determination. Testis determination is controlled by *Sry* (Sex-determining Region Y). *Sry* gene is male sex-determining gene on the Y chromosome (Martineau *et al.*, 1997). Expression of *Sry* transgenic in XX mice results in the initiation of testis cord formation (Koopman *et al.*, 1991; Eicher *et al.*, 1995). In the mouse, the *Sry* gene is expressed at 10.5 dpc and ceases after 12.5 dpc. *Sry* stimulates expression of genes such as *Sry* box9 (*Sox9*) which cause the differentiation of the Sertoli cell from coelomic epithelial cells (Karl and Capel, 1998). The Sertoli cell is the first cell to differentiate in the testis (Magre and Jost 1991). After differentiation, Sertoli cells move into the gonad and aggregate with primordial germ cells and start proliferate at 11.25 dpc in the mouse. Proliferation of Sertoli cells increases the size of testis and is determined by the expression of *Sry* (Schmahl *et al.* 2000; Schmahl and Capel 2003).

Induction of testicular cords in the testis

The fourth stage is induction of testicular morphology and seminiferous cord formation. Testicular cord formation is an important event in establishing the adult testis

morphology. In the mouse after Sertoli cell differentiation, formation of testicular cords and sex-specific vascularization occur around 11.5 -12 dpc and are complete by 12.5 dpc (Martineau *et al.*, 1997). First step in cord formation is aggregation of Sertoli cells with PGCs. The second step is the migration of mesonephric cells from the adjacent mesonephros to the differentiating testis (Cupp and Skinner 2005). Testis development is initiated by Sertoli cell differentiation and Sry expression. Testis failed to develop well-differentiated testis cords when mesonephros was removed at 11.5 dpc (Buehr *et al.*, 1993). Migration of mesonephric cells is required for seminiferous cord formation (Merchant-Larios *et al.*, 1993). It has been demonstrated that some paracrine growth factors such as Hepatocyte growth factor (HGF), Fibroblast growth factor 9 (Fgf9), neurotrophin-3 (NT3), VEGF and Platelet-derived growth factors (PDGFs) are involved in mesonephric cell migration and cord formation (Ricci *et al.* 1999; Colvin *et al.* 2001; Cupp *et al.* 2003; Bott *et al.*, 2006). In addition to surrounding the Sertoli-primordial germ cell aggregates to form testis cords, the migrating mesonephric cells may also initiate the formation of vasculature in the testis (Bott *et al.*, 2008). Formation of testicular cords and sex-specific vasculature are the key morphological markers to distinguish a testis from an ovary.

Development of a functional testis

The last step in testis morphogenesis is development of a functional testis. After seminiferous cord is formed, the other cells within the testis begin to differentiate. Sertoli cells produce the factors to initiate interstitial, peritubular, and Leydig cell differentiation (Cupp and Skinner, 2005). The peritubular cell surrounds and forms the exterior wall of

the seminiferous tubule. The peritubular and the Sertoli cells form the basement membrane surrounding the seminiferous tubule and their interactions are critical for germ cell development. The interstitial space outside the seminiferous tubules has the Leydig cells which are responsible for testicular steroid production (Skinner and Anway *et al.*, 2005). Estrogens and androgens have effects on testis growth, development of cell populations and differentiation of male reproductive tracts (Cupp and Skinner, 2005).

Spermatogenesis

Spermatogenesis is the process of sperm formation and maturation. It occurs in the seminiferous epithelium of the testis and is characterized by continuous germ cell maturation towards the center of the seminiferous tubules. During this time male germ cells undergo both mitotic and then meiotic divisions (Ruwanpura *et al.*, 2010). Specially, there is mitotic proliferation of spermatogonia, meiotic division of spermatocytes, differentiation of spermatids and final release of spermatozoa into the tubule lumen (Tripathi *et al.*, 2009). Gonocytes in the postnatal testis arise from PGCs and then develop to spermatogonial stem cells (SSCs). The foundation of spermatogenesis is SSC (Phillips *et al.*, 2010). Spermatogenic lineage development is referred to as the spermatogenic cycle (Clermont, 1972).

Spermatogonia are at the basement membrane of the seminiferous tubules. They are the primitive diploid germ cells (Phillips *et al.*, 2010). Undifferentiated type A spermatogonia differ in the topographical arrangement on the seminiferous tubule basement membrane and can be subdivided into A_{single} (A_s), A_{paired} (A_{pr}) and A_{aligned} (A_{al})

spermatogonia (Huckins, 1971). Differentiating spermatogonia (A1, A2, A3, A4 and B) are found at specific stages of the seminiferous epithelium (Oakberg, 1971). Type B spermatogonia develop to primary spermatocytes that then progress to meiosis. Two meiotic divisions lead to the formation of secondary spermatocytes and haploid spermatids respectively. Spermatids mature into spermatozoa which will be released from the seminiferous epithelium (Phillips *et al.*, 2010). During normal spermatogenesis, apoptosis regulates the ratio of Sertoli cells to germ cell number. Apoptosis induces programmed cell death to eliminate the germ cells which fail to replicate their DNA accurately during cell division (Ruwanpura *et al.*, 2010).

Hormonal regulation of spermatogenesis

The role of FSH in spermatogenesis

The size of the Sertoli cell population is set in early postnatal life and it is a major determinant of sperm output in the adult animal (Ruwanpura *et al.*, 2010). FSH increases the proliferation of Sertoli cells (Sharpe *et al.*, 2003); regulates the spermatogonial population in rodents (McLachlan *et al.* 2002) and has been demonstrated to be involved in spermatogonial survival (Beardsley & O'Donnell 2003; Ruwanpura *et al.* 2008a). An *in vivo* study demonstrated that FSH contributes to the proliferation of undifferentiated type A spermatogonia in immature testis cultures (Boitani *et al.*, 1993). If FSH is suppressed then there is an increase in spermatogonial apoptosis in immature and adult rats (Ruwanpura *et al.* 2008a, b, 2010). Besides its role in regulation of spermatogonia population, FSH also plays roles in meiosis and spermiogenesis. Vihko *et al.* reported

that FSH is critical for the meiosis in the first wave of rat spermatogenesis (Vihko *et al.*, 1991). Hypogonadal (hpg) mice expressing transgenic FSH showed that FSH supports spermatogonial proliferation and the stimulation of meiotic and post-meiotic germ cell development (Haywood *et al.* 2003).

The role of testosterone in spermatogenesis

In immature testes, testosterone is essential for gonadal maturation and testicular differentiation (Ruwanpura *et al.*, 2010). Somatic Sertoli cells transduce signals from testosterone into the production of factors that are necessary for germ cells when they become spermatozoa (Walker and Cheng 2005). Only Leydig cells, peritubular cells and Sertoli cells express androgen receptor (AR). No AR is expressed in germ cells of the mature testis (Walker and Cheng 2005). It is demonstrated that testosterone is able to suppress Sertoli cell proliferative activity *in vitro* (Buzzard *et al.*, 2003). Without testosterone, progressive germ cell degeneration begins during stage VII of the spermatogenic cycle (Walker and Cheng 2005). In the absence of androgen replacement, spermatogenesis is arrested during meiosis in Hpg male mice which lack both FSH and LH because of an inactivated mutation at the GnRH locus. Testosterone treatment also confers a qualitative recovery of spermatogenesis in Hpg mice (Holdcraft and Braun 2004). AR Sertoli cell knockout mice appeared arrested at late spermatocytes, indicating that the completion of meiosis and spermatogenesis is in need of androgen in the first spermatogenic wave (Chang *et al.* 2004; De Gendt *et al.* 2004). Testosterone not only plays roles in regulation of spermatogonia, but also is important for meiosis and spermiogenesis in rodents (McLachlan *et al.* 2002; Haywood *et al.* 2003).

Ovarian Development and Differentiation

The undifferentiated or bipotential gonadal ridge appears at approximately 10.5 dpc between the coelomic epithelium and the mesonephros in the mouse. The fate of the gonad is determined by both male-promoting and female-promoting signals. Sry, Sox9, and fibroblast growth factor 9 (Fgf9) initiate testis differentiation in indifferent gonads. Wingless-type member 4 (Wnt4) and R-spondin 1 (RSPO1) promote indifferent gonads into ovary differentiation (Fig. 2) (DiNapoli and Capel *et al.*, 2008). XX gonads do not have Sry, so the female promoting signals shut down the male signals and initiate ovarian development. The ovarian differentiation pathway involves the increase of the signaling of WNT4 by RSPO1, which up-regulates β -catenin. β -catenin up-regulates WNT4 and other proteins such as Follistatin (FST) (Edson *et al.*, 2009). FST inhibits formation of the XY-specific coelomic vessel in XX gonads. WNT4/FST signaling inhibits downstream events of testis development. FST also plays a critical role in the survival of germ cells in ovary.

Ovarian folliculogenesis

Autocrine, paracrine and endocrine factors contribute to ovarian folliculogenesis. Besides oocytes, granulosa cells and theca cells also develop and play critical roles in ovary. There are several steps in ovarian folliculogenesis: (1) formation of the primordial follicle, (2) Maintenance of primordial follicles and initial recruitment (3) preantral folliculogenesis, (4) theca formation and physiology, (5) antral follicle formation, (6) the

preovulatory follicle, cumulus expansion, ovulation, and luteinization (Fig. 3) (Edson *et al.*, 2009).

Formation of the primordial follicle

After migration of PGCs, oogonia undergo mitotic proliferation. Mouse germ cells are connected by intercellular bridges in the ovaries from 11.5 to 17.5 dpc (Pepling and Spradling *et al.*, 1998). Around 13.5 dpc in the female mouse, the first wave of oogonia begin meiosis and finally arrest as diplotene oocytes close to birth (Bristol-Gould *et al.*, 2006). Individual oocytes in these nests lack surrounding somatic cells and majority of the oocytes will undergo apoptosis. Cysts undergo apoptosis between 20.5-22.5 dpc in mice, during which only about one third of the oocytes survive to form primordial follicles (Pepling and Spradling, 2001, Edson *et al.*, 2009). Some oocytes survive after germ cell cluster breakdown. When those oocytes are individually surrounded by squamous pre-granulosa cells, primordial follicle formation occurs. This is the first stage of folliculogenesis and it occurs in the days immediately after birth in female mice (Edson *et al.*, 2009). During early postnatal period in the rodent, the primordial follicles assemble. Primordial follicles consist of one oocyte and an incomplete layer of squamous pre-granulosa cells. These primordial follicles are surrounded by stromal-interstitial cells but no apparent theca cell layers or organized mesenchymal tissue (Skinner, 2005).

Maintenance of primordial follicles and initial recruitment

Follicle recruitment is subdivided into two categories. The first one is initial activation of primordial follicles and it occurs throughout life until menopause. The

second one is cyclic recruitment of a limited number of small follicles from the growing cohort, from which a subset is selected for dominance and ovulation after puberty (Edson *et al.*, 2009). The majority of primordial follicles exist in a quiescent state in which the oocyte is arrested in prophase I of meiosis. In sexually mature animals, follicles leave the arrested pool and transform from primordial to primary follicles (Bristol-Gould *et al.*, 2006). Morphological changes in granulosa cells from squamous to cuboidal are the histological marker for the transition from primordial to primary follicle. Several transcription factors and signaling pathways regulate this early step in folliculogenesis. NOBOX (newborn ovary homeobox), SOHLH1 (spermatogenesis and oogenesis helix-loop-helix 1), and SOHLH2 are key transcription factors during the transition from primordial to primary follicles (Edson *et al.*, 2009). Transgenic mice lacking *Nobox*, *Sohlh1*, or *Sohlh2* have defects in the transition from primordial to primary follicle and are infertile (Choi *et al.*, 2008; Hao *et al.*, 2008; Pangas *et al.*, 2006; Rajkovic *et al.*, 2004). *Nobox*^{-/-}, *Sohlh1*^{-/-} *Sohlh2*^{-/-} mice have the similar numbers of germ cell clusters and primordial follicles as controls. However, by postnatal day 3, these transgenic mice lack primary follicles and experience an early postnatal loss of oocytes. It is reported that KIT ligand/KIT signaling contributes to the transition from primordial to primary follicle and induces the PI3K/AKT pathway which lead to phosphorylation and inactivation of forkhead box O3 (Foxo3). Foxo3 is a key oocyte factor critical for suppressing primordial follicle activation (Castrillon *et al.*, 2009).

Preantral folliculogenesis

Preantral folliculogenesis is characterized by oocyte growth, granulosa cell proliferation and acquisition of theca cell layer. Preantral follicle growth in mice starts at 10 to 12 days after birth when a cohort of developing follicles reaches the second stage of folliculogenesis. Secondary follicles contain oocytes in the middle of their growth stage which are surrounded by two or more layers of granulosa cells (Edson *et al.*, 2009). The communication between granulosa cells and oocytes is bidirectional and occurs throughout follicular development (Eppig *et al.*, 2002). Specifically, the oocyte depends on its relationship with accompanied somatic granulosa cells to support its growth and regulate the progression of meiosis. At the same time, oocytes stimulate granulosa cell proliferation, differentiation, and function. For example, Oocyte-secreted members such as the transforming growth factor β (TGF β) family, growth differentiation factor (GDF)-9, and bone morphogenic protein (BMP)-15 play critical roles in early follicular development (Dong *et al.*, 1996; Dube *et al.*, 1998; Elvin *et al.*, 1999). These oocyte-derived paracrine factors also stimulate follicular somatic cell proliferation and steroidogenesis (Elvin *et al.*, 1999; Solovyeva *et al.*, 2000).

Theca formation and physiology

Once the follicle develops two layers of granulosa cells, a layer of theca cells differentiate to the outermost layer (Hirshfield, 1991a). The theca interna is a layer of highly vascularized steroidogenic cells adjacent to the basal lamina, while the theca externa is a loosely organized band of non-steroidogenic cells between the theca interna

and the interfollicular stroma. The theca interna is composed of mitochondria with tubular cristae, smooth endoplasmic reticulum, and abundant lipid vesicles (Magoffin, 2005). The theca externa contains fibroblasts, smooth muscle-like cells, and macrophages, which are important during ovulation (Hirshfield, 1991b). Mouse follicles develop to the large antral stage containing fully grown oocytes by 18–24 days after birth (Eppig *et al.*, 2002).

Antral follicle formation

During antral folliculogenesis, multiple small fluid-filled spaces combine to form a single antral cavity. Two functionally distinct granulosa cell populations are separated by the antral cavity. At the wall of the follicle the newly formed mural granulosa cells are critical for steroidogenesis and ovulation while the antral granulosa cells surround the oocyte which promotes its growth and development (Edson *et al.*, 2009). FSH is essential for antral follicular development (Kumar *et al.*, 1997). FSH inhibits granulosa cell apoptosis and follicular atresia and is necessary for antral granulosa cell proliferation, estradiol production, and LH receptor expression (Chun *et al.*, 1996; Richards, 1994).

The preovulatory follicle, cumulus expansion, ovulation, and luteinization

The majority of follicle populations in the growing pool undergo atresia and only a few antral follicles in a developing cohort arrive at the preovulatory stage. Increased follicular estradiol production increases pituitary LH and eventually induce the LH surge. Preovulatory follicles produce luteinizing hormone/choriogonadotropin receptor (LHCGR) at high concentrations in granulosa cells which enable them to respond to the

LH surge. LH surge initiates downstream events leading to oocyte meiotic resumption, cumulus expansion, follicle rupture and final terminal differentiation of the remaining granulosa and theca cells to form the CL (Edson *et al.*, 2009). Ovulation is a complex LH-induced process that allows the release of an oocyte. The proper formation of an extracellular hyaluronan (HA) rich matrix by the cumulus oocyte complex (COC) is critical for ovulation. Genes induced by LH surge and essential for proper expansion are hyaluronan synthase 2 (HAS-2) and cyclooxygenase-2 (COX-2). They control the synthesis of hyaluronan and prostaglandins (Richards, 2005). After follicle rupture and release of the cumulus-oocyte complex, the remaining granulosa and theca cells differentiate to form the CL. The CL is an endocrine structure responsible for secreting progesterone to stimulate the uterus and maintain pregnancy (Edson *et al.*, 2009).

Vascular Endothelial Growth Factor

The *Vegf* family comprises five members, including *Vegfa*, *Vegfb*, *Vegfc*, *Vegfd* (*Figf*, c-fos-induced growth factor) and placenta growth factor (Pgf). The significant one of VEGF family is VEGFA (Tammela *et al.*, 2005). It is an endothelial cell-specific mitogen driven from arteries, veins and lymphatics *in vitro*. VEGFA is an important regulator of both developmental and tumorigenic angiogenesis (Grunstein *et al.*, 2000). It plays an essential role in embryonic vasculogenesis and angiogenesis in the mouse. It is also involved in skeletal growth and endochondral bone formation and angiogenesis in endocrine glands (Ferrara, *et al.*, 2003; Ferrara, 2004).

VEGFA gene

The human VEGFA gene is located on chromosome 6p21.3 and is organized in eight exons, separated by seven introns (Houck *et al.*, 1991; Tischer *et al.*, 1991; Vincenti *et al.*, 1996) and can be alternatively spliced to produce pro-angiogenic (angiogenic) or anti-angiogenic (inhibitory) isoforms by different factors (Fig. 4) (Harper and Bates, 2008). VEGFA is a heparin-binding homodimeric glycoprotein of 45 kDa (Ferrara and Henzel, 1989).

VEGFA isoforms

VEGFA isoforms are generated by alternate splice-site selection in the terminal exon (Nowak *et al.*, 2008). Alternative exon splicing results in proangiogenic or angiogenic polypeptides of 111, 121, 145, 148, 165, 183, 193, 189, and 206 and the anti-angiogenic or inhibitory polypeptides 189b, 183b, 165b, 145b, 121b, acids for human (Ferrara *et al.*, 1991; Woolard *et al.*, 2004; Harper and Bates, 2008). In rodent and domestic livestock, VEGFA isoform contains one less amino acid than the human VEGFA isoforms (Grunstein *et al.*, 2000). Alternate 5' and 3' splice site selection in exons 6, 7 and 8 generate multiple isoforms (Harper and Bates, 2008). Two mRNA isoform families are generated. Proximal splice-site selection (PSS) in exon 8 results in pro-angiogenic VEGFA_{xxx} isoforms (xxx is the number of amino acids), while distal splice-site selection (DSS) results in anti-angiogenic VEGFA_{xxxb} isoforms. SR protein splicing factors including ASF/SF2, SRp40 and SRp55 regulate the VEGFA splicing. Both

ASF/SF2 and SRp40 favored PSS, but SRp55 upregulated VEGFA_{xxx}b (DSS) isoforms (Nowak *et al.*, 2008).

Angiogenic isoform

VEGFA angiogenic isoforms have the exon 8a (Nowak *et al.*, 2008). VEGFA165 (VEGFA164 in rodent) was the first VEGFA isoform discovered in humans (Ferrara and Henzel, 1989). VEGFA164 is the predominant VEGFA angiogenic isoform. It is a multifunctional cytokine that is necessary for normal vascular development. It has potential vascular permeable activity, stimulates endothelial cell migration and is mitogenic for endothelial cells. It also re-programs endothelial cell gene expression and is an endothelial cell survival factor (Nagy *et al.*, 2003). VEGFA165 lacks the residues encoded by exon 6, however, VEGFA121 does not have the residues encoded by exons 6 and 7 (Ferrara, 2004). In mouse, due to the possession of exon 7, VEGFA164 results in increased heparin-binding affinity compared to VEGFA120. VEGFA188, containing both exons 6 and 7, leads to a dramatic increase in charge and extracellular matrix (ECM) association compared to the VEGFA164 and VEGFA120 (Grunstein *et al.*, 2000).

Anti-angiogenic isoform

VEGFA anti-angiogenic or inhibitory isoforms have the exon 8b (Nowak *et al.*, 2008). In 2002, Bates *et al.* identified a VEGFA_{xxx}b isoform: VEGFA165b in human (in rodent VEGFA164b). They found that VEGFA165b inhibits VEGFA165-mediated proliferation, migration of endothelial cells and vasodilatation of mesenteric arteries. The results of their research suggest that VEGF165b may be anti-angiogenic. VEGFA_{xxx}b

isoforms are expressed in many tissues (Bates *et al.*, 2002). VEGFA_{xxx}b isoforms bind to VEGFR2 (KDR), but lead a poorly activated kinase and weak downstream signal transduction. VEGFA₁₆₅b does not increase chronic microvascular permeability *in vivo*, and reduces conditionally immortalized human glomerular endothelial cell monolayer permeability *in vitro*. VEGFA₁₆₅b also inhibits angiogenesis *in vivo* in the rat mesentery. Moreover, VEGFA₁₆₅b suppresses physiological angiogenesis in mammary tissue in transgenic mice and pathological angiogenesis in murine tumour models (Harper and Bates, 2008). All these studies suggest that VEGFA_{xxx}b isoforms have an opposite function compared to VEGFA angiogenic isoforms.

VEGFA receptors

VEGFA binds two related tyrosine kinases receptors: FMS-like tyrosine kinase1 (FLT1, also known as VEGFR1) and kinase insertion domain protein receptor (KDR, also known as VEGFR2 and FLK1). The major mediator of the mitogenic, angiogenic and permeability effects of VEGFA is KDR. Also KDR is involved in mediating endothelial cell proliferation, survival and vascular permeability. In contrast, FLT1 may play an inhibitory role by sequestering VEGFA and preventing its interaction with KDR. In addition to these tyrosine kinases receptors, VEGFA also interacts with a family of coreceptors: the neuropilins (Ferrara, 2004).

FLT1

Although Flt1 was the first VEGFR to be identified, the precise function of this molecule is unclear. Recent evidence indicates that the controversial reports may be due

to the fact that Flt1 functions and signaling properties are dependent on the developmental stage and the cell type such as endothelial vs. hematopoietic cells (Ferrara, *et al.*, 2003; Ferrara, 2004). In response to VEGFA, Flt1 reveals a weak tyrosine autophosphorylation (de Vries *et al.*, 1992, Waltenberger *et al.*, 1994). One study reported that Flt1 activation causes inhibition of VEGFR2-dependent endothelial cell proliferation and the pathway is PI3 kinase dependent (Zeng *et al.*, 2001). Other studies indicated that Flt1 interacts with different signal-transducing proteins and generate a mitogenic signal (Landgren *et al.*, 1998; Maru *et al.*, 1998).

KDR

VEGFA angiogenic isoforms act primarily through VEGFR2 or KDR to initiate multiple downstream signal pathways. VEGFA angiogenic isoforms stimulate robust phosphorylation of tyrosine 1175, resulting in the activation of phospholipase C γ (Fig. 5) (Harper and Bates, 2008). Phospholipase C γ activates protein kinase C which results in the activation of Ras or Raf, mitogen-activated protein kinase (MAPK) and extracellular-signal-regulated kinase-1/2 (ERK1/2). The result of ERK phosphorylation is endothelial cell proliferation. PLC γ also activates PKC via diacylglycerol (DAG) and then increase concentrations of intracellular calcium (Shu *et al.*, 2002). Besides PLC γ , the adaptor molecule Shb binds to phosphorylated Tyr1175 of KDR. Tyr1173/1175 is coupled to VEGFA-induced PI3K activation. PI3K then activates the serine/threonine kinase AKT/PKB and mediates survival of the endothelial cells. AKT/PKB also regulates nitric oxide (NO) production by phosphorylation and activation of endothelial NO synthase (eNOS). eNOS regulates vascular permeability. The p38MAPK and FAK pathways are

also postulated to affect actin reorganization and focal adhesion turnover to influence migration. Heat-shock protein-27 (HSP27) is phosphorylated by p38MAPK. It is a molecular chaperone that positively regulates VEGFA-induced actin reorganization and migration. Several of these VEGFA signal transduction pathways may interact with each other to initiate endothelial cell differentiation, migration, proliferation and survival (Olsson *et al.*, 2006).

Activation of KDR is phosphorylated by binding VEGFA anti-angiogenic isoforms; however different tyrosine residues show that it may not simply be an inactive competing inhibitor. Phospho-peptide mapping and site-specific phospho-antibody experiments show that VEGFA anti-angiogenic isoforms only partially activate KDR suggesting a partial intracellular rotation. This results in a rapid closure of the ATP binding site and fast inactivation that lead to a poorly activated kinase and weak, transient phosphorylation of ERK1 and ERK2 (Fig. 5) (Harper and Bates, 2008). This suggests that VEGFA anti-angiogenic isoforms initiate a poor and weak downstream signal.

The Role of VEGFA in gonads

The role of VEGFA in the ovary

Angiogenesis is a key aspect of normal cyclical ovarian function. Both follicular growth and the development of the CL are dependent on the proliferation of new capillary vessels (Ferrara, 2004). It is demonstrated that the VEGFA mRNA expression is also temporally and spatially associated with the proliferation of blood vessels in the ovary (Phillips *et al.*, 1990; Ravindranath *et al.*, 1992). VEGFA stimulates

neovascularization, regulates vascular permeability and is one of the key factors that regulate angiogenesis in the ovary (Kaczmarek *et al.*, 2005).

Follicle activation is related to increased VEGFA and increased blood vessel extension (Mattioli *et al.*, 2001). One *in vitro* study showed that direct ovarian administration of VEGFA significantly increased the number of preantral follicles in the rat ovary suggesting that VEGFA regulates the early follicle growth (Danforth *et al.*, 2003). Other research has demonstrated that endogenous VEGFA is essential for primordial follicle survival in the rodent (Roberts *et al.*, 2007). Iijima *et al.*, demonstrated that VEGFA angiogenic isoform promoted ovarian follicular angiogenesis, stimulated follicular development and increased the numbers of oocytes ovulated in mature cycling rats (Iijima *et al.*, 2005).

VEGFA promoted the primary into secondary follicle transition in bovine follicles activated *in vitro*. Thus, VEGFA plays a stimulatory role in early preantral follicular growth in cattle (Yang and Fortune *et al.*, 2007). In nonhuman primates, inhibition of VEGFA and KDR either suppresses follicular development or prevents ovulation (Zimmermann *et al.*, 2001; Zimmermann *et al.*, 2002). Zimmermann's research showed that injection of VEGFA antagonist into the pre-ovulatory follicle in rhesus monkey caused half of the animals not to ovulate. This indicates VEGFA may also stimulate ovulation in addition to follicle progression (Zimmermann *et al.*, 2002).

Our laboratory's finding on the role of VEGFA in the ovary

Recently, our laboratory published some findings about the role of VEGFA in the ovary. We demonstrated that VEGFA and KDR were expressed in pregranulosa and granulosa cells of all follicle stages and in theca cells of advanced-stage follicles. Rat ovaries treated with VEGFA receptor signal transduction inhibitor had a 94% reduction in vessel density. And these ovaries had more primordial follicles, fewer early primary, transitional, and secondary follicles, and greater total follicle numbers compared with control ovaries. The results indicated that VEGFA is involved in primordial follicle activation, maturation and survival (McFee *et al.*, 2009). Another study showed that rat ovaries treated with VEGFA164 or VEGFAxxxB antibody had vascular development increased by 93%. VEGFA164 treated ovaries had fewer primordial follicles and more developing follicles than controls. Ovaries treated with VEGFAxxxB antibody had fewer primordial and earlier primary follicles and more primary, transitional, and secondary follicles compared with controls. The data suggested that VEGFA proangiogenic isoform promotes follicular development (Artac *et al.*, 2009).

The role of VEGFA in the testis

Overexpression of VEGFA164 in the testis and epididymis of transgenic mice resulted in upregulation of VEGFA receptors in the endothelial cells, certain spermatogenic cells and the Leydig cells and caused infertility. The VEGFA164 overexpression in testis and epididymis resulted in spermatogenic arrest. This indicated that VEGFA may regulate male fertility (Korpelainen *et al.*, 1998). One study on male

roe deer showed the strength of VEGFA angiogenic isoforms mRNA expression is dependent on the season. It reached its highest level at the peak of spermatogenesis during the pre-rutting period and had its nadir at the end of the rut when involution already began. These results suggest that VEGFA angiogenic isoforms may directly affect the regulation of spermatogenesis (Wagener *et al.*, 2010).

Our laboratory's finding on the role of VEGFA in the testis

Our laboratory has demonstrated that VEGFA is critical for both vascular development and testicular cord formation. Both Treatment with Je-11 (a VEGFA antagonists) and VEGFR-TKI (a VEGFA receptor signal transduction inhibitor) perturbed cord formation and inhibited vascular density in rat cultured testis. Rat E13 testes were treated with a phosphoinositide 3-kinase (PI3K) pathway inhibitor which resulted in inhibition of both vascular density and cord formation. These studies suggest that VEGFA potentially works through the PI3K pathway to promote testicular cord formation and vascular density during testis morphogenesis (Bott *et al.* 2006). Treatment of E13 mouse organ culture testes with VEGFA angiogenic isoform or an antibody to antiangiogenic isoforms (anti-VEGFAxxxB) resulted in less organized and poorer seminiferous cords compared with controls. In addition, VEGFA angiogenic isoform and anti-VEGFAxxxB also increased the vasculature density in the mouse culture testes. The results from these studies suggest that an imbalance in the production of VEGFA pro-versus anti-angiogenic isoforms may impair testis morphology and development. Both VEGFA angiogenic and inhibitory isoforms are important for development of vasculature and seminiferous cords in rat testes (Baltes-Breitwisch *et al.*, 2010). Recently, we

demonstrated that KDR was expressed in germ cells E16, E17, P4, and after P20 in mouse testis. KDR-LacZ testis organ cultures treated with VEGFR-TKI had reduced numbers of KDR-LacZ stained cell in developing testes; this indicates decreased migration of KDR-expressing endothelial cells from the mesonephros to the testis. Thus, the VEGFA signal transduction inhibitor reduced the endothelial cell migration during testis formation (Bott *et al.*, 2010). Taken together, these studies suggest that VEGFA and its receptor are involved in the development of vasculature and seminiferous cords in both rat and mouse testes.

Conclusion

Vascular endothelial growth factor is one of the key factors regulating angiogenesis in the ovary. It has been demonstrated to be a potential regulator of follicle development and ovulation (Kaczmarek *et al.*, 2005; Danforth *et al.*, 2003). Since VEGFA is produced in numerous ovarian compartments and its important role in ovarian vascular physiology, we hypothesized that production of VEGFA by granulosa cell is critical for normal ovary morphogenesis and function. The objective of my first project is to determine the *in vivo* effects of granulosa-cell specific VEGF loss on ovary function and morphogenesis.

Besides the role in ovary, VEGFA also plays a critical role in testis development. VEGFA and its receptor KDR are present during testis morphogenesis. It has been demonstrated that VEGFA is involved in the development of vasculature and seminiferous cords in rodent testes and supports germ cell survival (Bott *et al.*, 2006, Caires *et al.*, 2009; Baltes-Breitwisch *et al.*, 2010). We hypothesized that VEGFA,

produced by Sertoli cells, plays an important role in testis morphogenesis and function.

The objective of my second project is to determine the *in vivo* effects of Sertoli cell-specific VEGFA loss on male reproductive organs' function and morphogenesis.

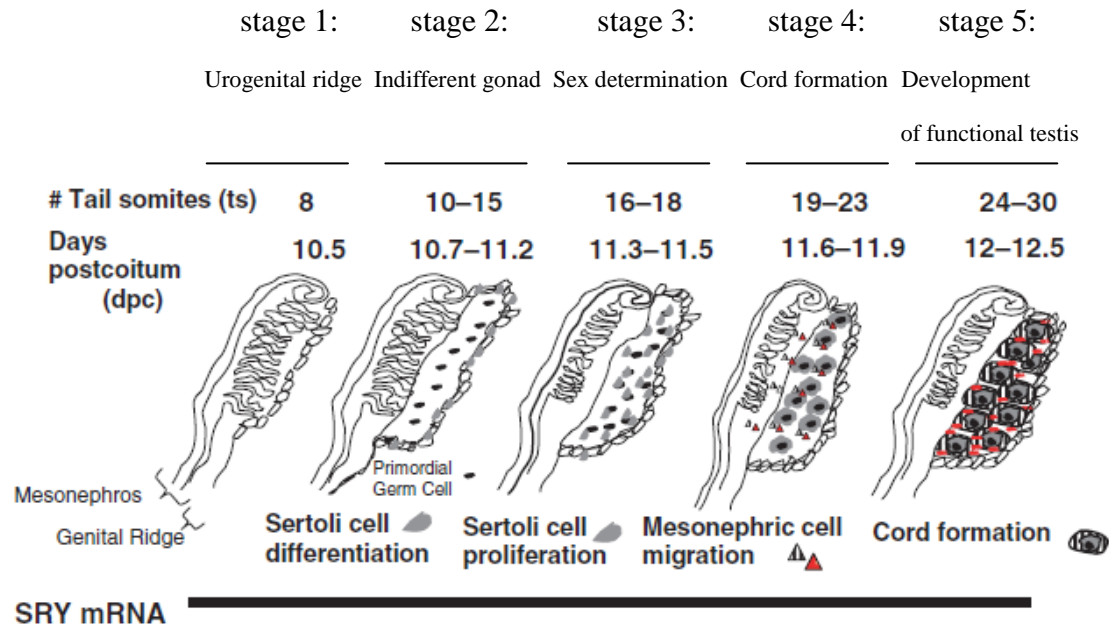


Fig.1 Morphology stages that occur during testis differentiation from genital ridge formation to cord formation and cell proliferation in the mouse with stage in number of tail somites (ts) and dpc (figure adapted from Cupp and Skinner, 2005)

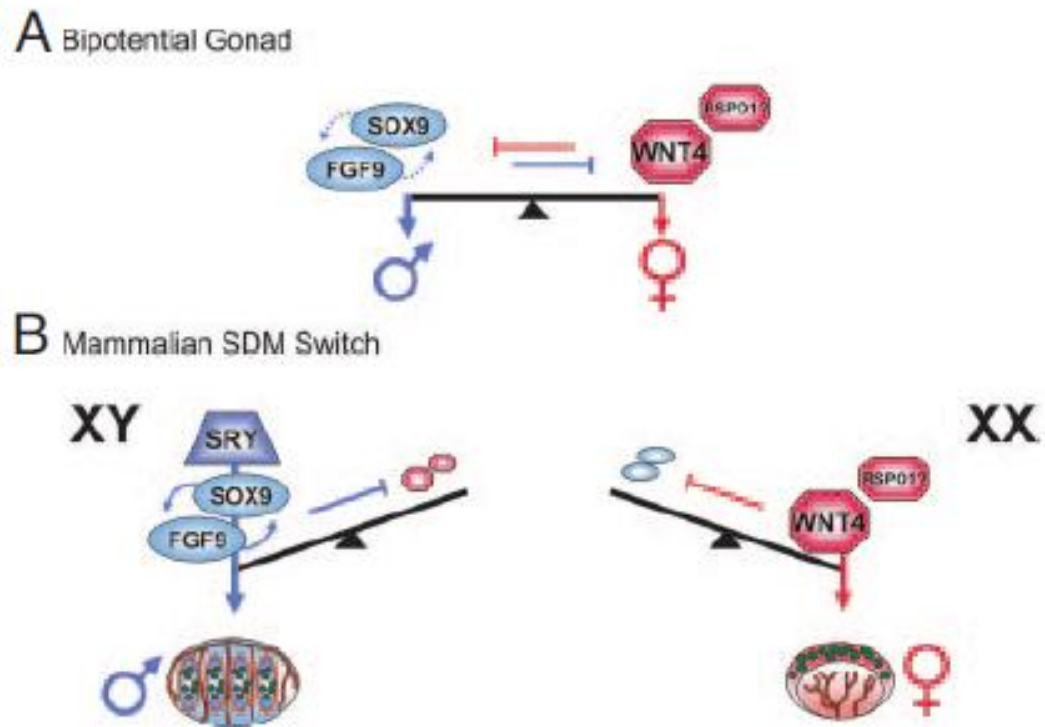


Fig.2 Model of Opposed Signals in Mammalian Sex Determination (figure adapted from DiNapoli and Capelm, 2008)

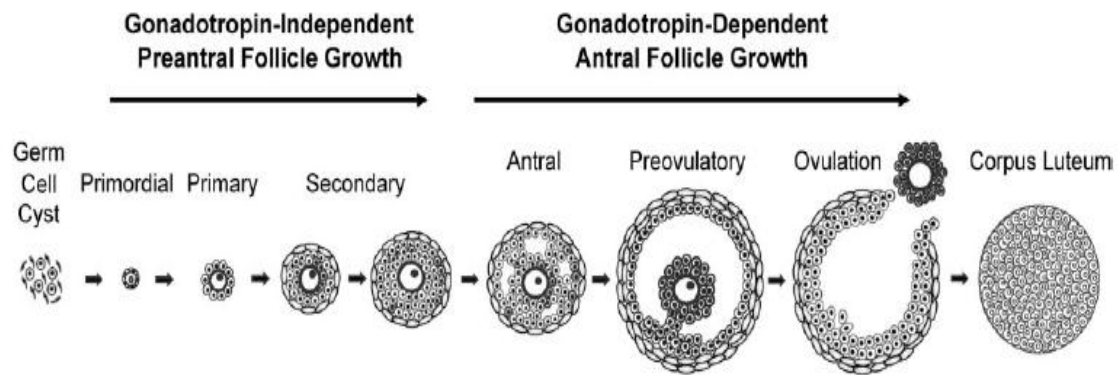


Fig.3 Major stages of mammalian folliculogenesis (figure adapted from Edson *et al.*, 2009).

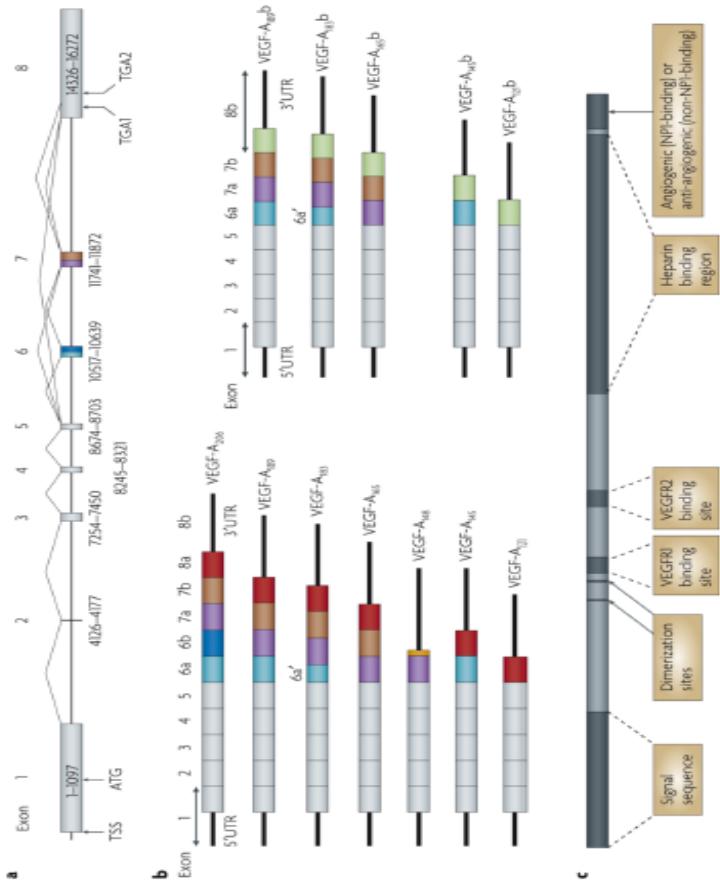


Fig.4 Protein and mRNA products of human VEGF-A folliculogenesis (figure adapted from Harper and Bates, 2008).

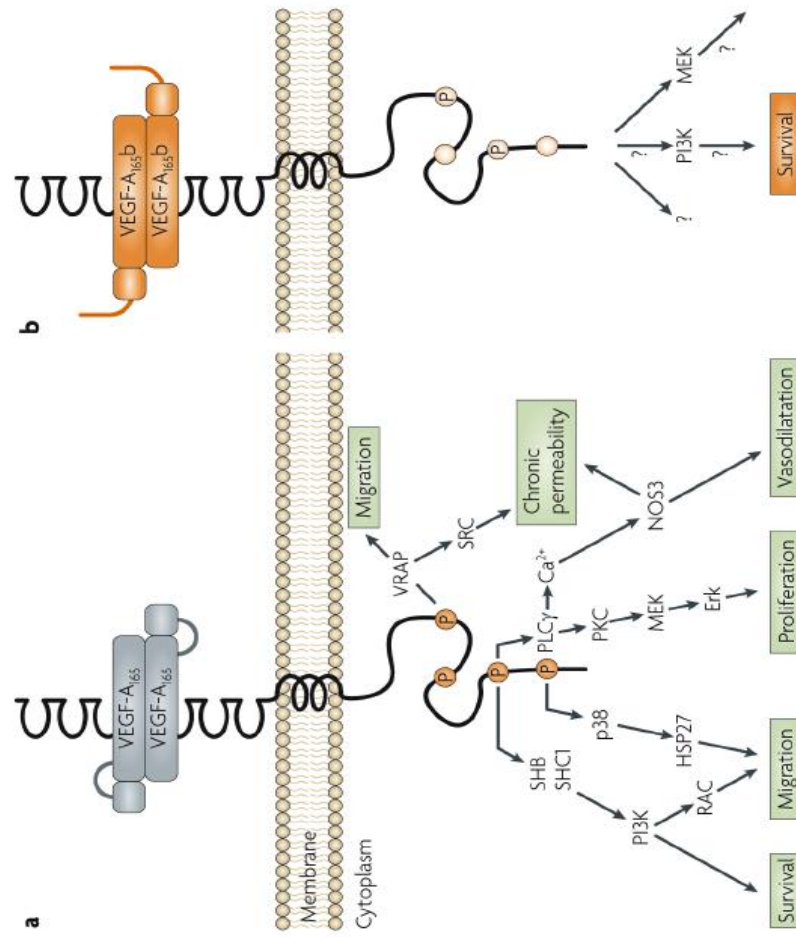


Fig.5 Signalling pathways downstream of vascular endothelial growth VEGF angiogenic and inhibitory isoforms (figure adapted from Harper and Bates, 2008)

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Chapter 2

Granulosa cell-specific Vascular Endothelial Growth Factor A (VEGFA) loss using pDMRT-1 Cre alters ovarian morphogenesis, reduces plasma estrogen concentrations and impairs fertility

Abstract

VEGFA is a critical regulator of vascular physiology in the ovary and plays an important role in the regulation of follicular growth. The objective of the current study was to evaluate effects of granulosa cell-specific production of VEGFA isoforms on the vascular development and ovarian morphogenesis. The *pDmrt1* gene is expressed in the indifferent gonad in precursor granulosa and Sertoli cells and in some specific germ cell lineages. We used a pDmrt1-cre mouse crossed to a floxed VEGFA mouse to determine potential phenotypes in female granulosa-cell (VEGFA-DGranG) specific knockouts. Ovaries, uterus, kidneys and adrenals were collected from adult mice, and weighed (control n=11, VEGFA-DGranG n=10). The weight of ovaries from VEGFA-DGranG was significantly smaller than that of the control (0.0144 ± 0.0014 VS 0.0183 ± 0.0009 g, $P < 0.05$). Ovaries from VEGFA-DGranG had reduced numbers of CL/ovary than the control (2.25 ± 0.4119 VS 4 ± 0.4629 , $P < 0.05$) and two of those VEGFA-DGranG females had abnormal CL morphology. Serum estradiol concentrations in the VEGFA-DGranG mice were 55% of control mice (48.83 ± 9.58 VS 89.48 ± 13.17 pg/ml, $P < 0.05$). The number of days mated until parturition was 12 days longer in VEGFA-DGranG X VEGFA-DSertG pairs versus control females mated with control males (38.27 ± 4.65 VS

26.18 \pm 2.17 days, $P < 0.05$). Therefore, we conclude from these data that absence of both VEGFA pro-angiogenic and anti-angiogenic isoforms may alter ovarian morphogenesis, follicle development and ovulation resulting in impaired fertility in female mice.

Introduction

In mammals, female reproductive life span depends not only on the size of the primordial follicle pool, but also on the complement of follicles and development of those follicles during the peripubertal period. Abnormalities in either primordial follicle pool development or follicle progression may result in impairment of ovarian function (Mazaud *et al.*, 2002). There is intense angiogenesis and increased permeability of blood vessels during follicular development, ovulation and formation of the corpus luteum. The availability of an adequate vascular supply to provide endocrine and paracrine signals have been shown to play a key role in the regulation of follicle growth (Redmer and Reynolds, 1996). Angiogenesis plays an important role in follicular development with independent regulation of vasculature within each follicle contributing to its development (Fraser, 2006). VEGFA is one of the key factors regulating angiogenesis in the ovary. VEGFA stimulates neovascularization, vascular permeability, acts as a survival factor and can also stimulates proliferation of vascular and nonvascular cells.

Several studies have demonstrated that VEGFA is important in the growth of capillary vessels around follicles in the ovary and it also promotes development of ovarian follicles in rats and mice (Shimizu *et al.*, 2003; Kaczmarek *et al.*, 2005; Danforth *et al.*, 2003; Quintana *et al.*, 2004), including both preantral and antral follicular

development (Shimizu *et al.*, 2007; Danforth *et al.*, 2003). VEGFA production is critical in the control of follicle growth and activity (Mattioli *et al.*, 2001). Kosaka *et al.* reported that VEGFA inhibited bovine granulosa cell apoptosis. These results demonstrated that VEGFA not only acts in vascular formation but also functions as a survival factor for ovarian follicles (Kosaka *et al.*, 2007).

In addition to development of the vasculature and apoptosis, VEGFA also appears to regulate ovulation. *In vivo* studies showed that inhibition of VEGFA and VEGF receptor-2 can inhibit follicular development or prevent ovulation (Zimmermann *et al.*, 2001; Zimmermann *et al.*, 2002). Furthermore, injection of a VEGFA antagonist into the pre-ovulatory follicle in rhesus monkey resulted in half of the animals failing to ovulate with the large follicles of these females becoming luteinized (Zimmermann *et al.*, 2002). Thus, in addition to follicular development VEGFA also appears to affect ovulation.

The *Vegf* family comprises five members, including *Vegfa*, *Vegfb*, *Vegfc*, *Vegfd* (officially called *Figf*, c-fos-induced growth factor) and placenta growth factor (*Pgf*). The significant one of *Vegf* family is *Vegfa* in the ovary. The *Vegfa* gene has eight exons and can be alternatively spliced by different factors to produce angiogenic or inhibitory isoforms. VEGFA angiogenic or inhibitory isoforms have opposite functions (angiogenic isoforms promote vascular development while anti-angiogenic isoforms inhibit vascular development). To initiate biological effects, VEGFA binds two related tyrosine kinase receptors: FMS-like tyrosine kinase 1 (FLT1, also known as VEGFR1) and kinase insert domain receptor (KDR, also known as VEGFR2 and FLK1). The primary receptor is KDR (Ferrara *et al.*, 2003). KDR is the major mediator of the mitogenic, angiogenic and

permeability enhancing effects of VEGFA. KDR is involved in mediating endothelial cell proliferation, survival and vascular permeability, whereas FLT1 might play an inhibitory role by sequestering VEGFA, and preventing its interaction with KDR (Ferrara *et al.*, 2004). Roberts *et al.* reported ovarian administration of antibodies to KDR resulted in significant depletion of primordial follicle numbers, whereas anti-FLT1 antibodies did not (Roberts *et al.*, 2007). This is consistent with KDR receptor as the primary mediator of VEGFA action in the ovary and that the FLT1 receptor might be a decoy for VEGFA.

In the human ovary, VEGFA is produced by the theca and granulosa cells (Yamamoto *et al.*, 1997; Roberts *et al.*, 2007). Our laboratory has demonstrated that VEGFA and its receptors are also localized to pre-granulosa and granulosa cells of all follicle stages and to theca cells of advanced-stage follicles in rat (McFee *et al.*, 2009). An *in situ* study showed the *in vivo* expression of VEGFA in different cell types including theca, cumulus, granulosa and luteal cells in mouse ovary (Shweiki *et al.*, 1993). Independent of its angiogenic effects, VEGFA may also be important in the regulation of follicle growth with direct effects on granulosa cells. Rat ovaries treated with VEGFA receptor-tyrosine kinase inhibitor had vascular development reduced by 94% and follicle progression was blocked since there were more primordial follicles, and fewer early primary, transitional, and secondary follicles compared to controls. This study demonstrated a novel role for VEGFA in the recruitment of primordial follicles into the growing follicle pool, as well as a potential survival factor for primary and later-stage follicles through vascular dependent and vascular-independent mechanisms (McFee *et al.*, 2009). Further studies from our laboratory demonstrated that treatments with both

neutralization of anti-angiogenic VEGFA isoforms and pro-angiogenic isoform VEGFA 164 enhanced vascular and follicular development in perinatal rat ovaries. These results suggest that VEGFA isoforms are involved in vascular development and follicle activation in the rat ovary with pro-angiogenic isoforms stimulating follicle progression and anti-angiogenic isoforms inhibiting or arresting follicle development (Artac *et al.*, 2009).

Thus, VEGFA is an important regulator of ovarian vascular physiology and is produced in numerous ovarian compartments. In the current study, we hypothesized that production of VEGFA isoforms (both pro and anti-angiogenic) by granulosa cells is critical for normal ovary morphogenesis and function. Therefore, the objective of present study was to determine the *in vivo* effects of granulosa-cell specific VEGFA loss on ovary function and morphogenesis.

Materials and methods

Animals

Granulosa cell-specific VEGFA KO mice were obtained by using the Cre-loxP approach. We used a line of VEGFA^{loxP/loxP} mice (Gerber *et al.*, 1999) from Napoleon Ferrara at Genentech to mate with pig Dmrt1-cre mice (Boyer *et al.*, 2002) to develop granulosa and Sertoli cell-specific VEGFA knockout mice (breeding scheme in Appendix A). The Dmrt1 gene is expressed in the indifferent gonad at 10.5 dpc in precursor cells that differentiate into Sertoli and granulosa cells. By 12.5 dpc, Dmrt1 is expressed in Sertoli cells and germ cells in XY gonads and in ovarian somatic cells and germ cells.

Dmrt1 is evident in Sertoli cells at all ages examined and absent in ovarian somatic cells from 13.5 dpc. Expression of Dmrt1 in primordial germ cells increases through 13.5 dpc and absent by 15.5 dpc. Sexual differences in germ cell Dmrt1 were detected after birth, when it was detected only in spermatogonia of the testis (Lei *et al.*, 2007). The pDmrt1 gene is expressed similar to mouse Dmrt1 (Boyer *et al.*, 2002). In our VEGFA-DGranG mouse, VEGFA gene exon 3 was flanked by two loxp sites and Cre, driven by pig Dmrt1, bound and deleted the DNA sequence between the two flox. The VEGFA was not expressed in the cells where Dmrt1 is present. We used Cre-negative VEGFA^{loxp/loxp} or VEGFA^{loxp/+} mice as controls and Cre-positive VEGFA^{loxp/loxp} mice as KO (VEGFA-DGranG). Ovaries, uterus, kidneys and adrenals were collected from female adult mice that were cycling, non-pregnant and were at random stage of the estrous cycle (control n=11, VEGFA-DGranG n=10) and weighed. All the female were approximately 250 days of age. All animal protocols were approved by the University of Nebraska Institute of Animal Care and Use Committee (IACUC) in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Genotyping:

DNA was extracted from tail samples and used for genotyping via polymerase chain reaction (primers and PCR programs are listed in Appendix B). Genomic DNA was extracted from tails of mice by proteinase K digestion and followed by 6M NaCl extraction and ethanol precipitation (Bott *et al.*, 2010).

Embedding, Histology and staining (hemotoxylin and eosin staining; H&E):

Ovaries were fixed in Bouin's solution and rinsed in 70% ethanol. Embedding was performed according to the standard procedures (McFee *et al.*, 2009; Bott *et al.*, 2010). Prior to paraffin embedding, ovaries were rinsed in 100% ethanol 1 hour for 3 times and CitriSolve (Fisher Scientific, Fairlawn, IL) 1 hour for 3 times. Embedded tissues were sectioned (5 μ m) and the slides were placed in an incubator (37 °C) overnight. Sectioned ovaries were deparaffinized, stained with hematoxylin for 1 min (VWR International, West Chester, PA) and eosin for 30 min (Ricca, Arlington, TX), dehydrated and mounted.

Immunohistochemistry:

Sectioned tissues were deparaffinized, rehydrated, and microwaved in 0.01 M sodium citrate to boil for 15 min. Then, tissues were cooled for 1 h and rinsed in 3% hydrogen peroxide in methanol for 20 min. Sections were blocked with 10% normal goat serum in PBS for 30 min at room temperature and washed in PBS 3 times. Immunohistochemistry was performed according to the standard protocol in our laboratory (Mcfee *et al.*, 2009). The Cyp11a1 primary antibody (catalog number: ab78416; Abcam, Cambridge, Mass., USA) was diluted in 1:200 in 10% normal goat serum. As a negative control, serial sections were processed without primary antibody. Biotinylated goat anti-rabbit secondary antibodies were diluted 1:300. Secondary antibody was detected using aminoethyl carbazole (AEC) chromagen substrate solution (ZYMED Laboratories, San Francisco, CA). Sections were counterstained with hematoxylin to determine cell types that were stained with respective antibodies.

RNA isolation:

Total RNA from ovaries was extracted using Tri-Reagent according to manufacturer's protocol (Sigma, St. Louis, MO). After isolation total RNA was dissolved in 20 μ l diethyl pyrocarbonate (DEPC) water. The extracted RNA was diluted 1:14 in DEPC water and the concentration was determined by spectroscopy at 260nm using the Lambda EZ 150 (Perkin Elmer).

Reverse Transcription (RT)-PCR:

Before RT-PCR, 1 μ l of RNA was removed from each sample to a new tube with 15 μ l DEPC water to be used as "No RT" to detect the residual genomic DNA. RT was performed on 5 μ g of template using SuperScript II (Invitrogen, Carlsbad, CA) according to the manufacturer's recommended protocol (McFee *et al.*, 2009; Bott *et al.*, 2010). RT samples were incubated in 65°C with 2 μ l of oligo (dT) 15 primer (Promega), 2 μ l dextoxynucleotide triphosphates (Fermantas; 10 mM dNTP mix), 3.8 μ l DEPC water, and then immediately chilled on the ice. After that, 1 μ l SuperScript II reverse transcriptase (200 units/ μ l; Invitrogen, Carlsbad, CA), 3.2 μ l of stand buffer (Invitrogen, Carlsbad, CA), 2 μ l 0.1 M DL-dithiothreitol (DTT) (Invitrogen, Carlsbad, CA), 1 μ l DEPC water were added into each sample. The samples were incubated at 42°C for 1 hour followed by 15 min at 70°C to inactivate the reverse transcriptase. The cDNA was stored at 20°C for subsequent real time-PCR.

Quantitative RT- PCR (QRT-PCR):

Quantitative real-time PCR primers and probes used in the experiment were shown in table 1. Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) gene is a housekeeping gene and used as a control for RNA amplification. QRT-PCR was performed in 364 well plates (Axygen Scientific, Union City, CA) with an adhesive cover film (VWR, Scientific Products, North Kansas City, MO). *Gapdh*, *Bcl-2* and *Bax* were performed with 10 μ l Taqman universal Master Mix (Applied Biosystem, Foster City, CA), 1 μ l cDNA, 0.5 μ l of each forward and reverse primers, 2 μ l probe, and 6 μ l DEPC to make up the reaction volume to 20 μ l. *Foxo3a*, *Gdnf-9*, *Cyp17a1* and *Cyp11a1* were performed with 10 μ l SYBR Green PCR Master Mix (Applied Biosystem, Foster City, CA), 1 μ l cDNA, 0.6 μ l of each forward and reverse primers, and 7.8 μ l DEPC to make up the reaction volume to 20 μ l. Experimental and *Gapdh* PCRs were carried out in separate wells in triplicate. An arbitrary value of template was assigned to the highest standard and corresponding values were assigned to the subsequent dilutions. These relative values are plotted against the threshold value for each dilution to generate a standard curve. The relative amount for each experimental and *Gapdh* triplicate was assigned an arbitrary value based on the slope and y-intercept of the standard curve. The average of the experimental triplicate is divided by the average of the *Gapdh* triplicate, and the resulting normalized values are used for statistical analysis (McFee *et al.* 2009).

Blood collection and Hormone Enzyme-Linked Immunosorbent Assay (ELISA):

Blood was collected at the time of euthanization and put into tubes with 20 μ l EDTA solution (Fisher Scientific Co., Fair Lawn, NJ). Blood sample was immediately centrifuged at 1250 X g for 10 mins, the plasma was removed into a new tube and stored in -80°C. Estradiol level was determined by Estradiol ELISA kit (Alpha Diagnostics International Inc., San Antonio, TX; Cat. No.1920). Samples remained undiluted and were run according to the manufacture's direction. Standard curve samples were made of 10 pg, 50 pg and 150 pg to ensure that all samples were on the curve. Assay sensitivity was 10 pg/ml. The progesterone concentration was determined by Progesterone ELISA kit (Alpha Diagnostics International Inc., San Antonio, TX; Cat. No. 1860). Standard curve samples were made of 0.5 ng/ml, 1 ng/ml, 5 ng/ml, 10 ng/ml, 30 ng/ml. Assay sensitivity was 0.2 ng/ml. Testosterone concentration was determined by Testosterone ELISA kit (Alpha Diagnostics International Inc., San Antonio, TX; Cat. No.1880). Standard curve samples were made of 0 ng/ml, 0.5 ng/ml, 1 ng/ml, 2.5 ng/ml, 10 ng/ml and 20 ng/ml. Assay sensitivity was 0.125 ng/ml.

Statistical analysis:

All data were analyzed by two-sample *t*-test using SAS software (SAS institute, Cary, NC). Differences in data were considered statistically significant at $P < 0.05$.

Results

Phenotypes in the female ovary of VEGFA-DGranG mice:

The ovarian weight and morphology were used to evaluate the effect of granulosa cell-specific VEGFA loss on female ovary. As shown in figure 2A, the weight of ovaries was significantly decreased for VEGFA-DGranG females compared with the controls (0.0144 ± 0.0014 VS 0.0183 ± 0.0009 g, $P < 0.05$). To assess the effect of granulosa cell-specific VEGFA loss on ovary histological morphology, ovaries were stained by H&E. VEGFA-DGranG mice had ovaries with fewer antral follicles and corpus lutea (CL), and two VEGFA-DGranG mice had abnormal morphology in structures that appeared to be CL (fig. 1). To examine the effect of granulosa cell-specific VEGFA loss on ovulation, we counted the number of CL in the ovary. Cyp11a1 was used as a marker to count numbers of CL/ovary (fig. 3A, B, C). As shown in figure 3D, the number of CL/ovary from VEGFA-DGranG mice was significantly less than that from control mice (2.25 ± 0.4119 VS 4 ± 0.4629 , $P < 0.05$). These results indicated that there were reduced ovulation rate potentially caused by reductions in production of antral follicles on the ovary of VEGFA-DGranG mice.

Effect of Granulosa cell-specific VEGFA loss on female mouse uterus, kidneys and adrenals:

To test whether Granulosa cell-specific VEGFA loss affects other organs in the mouse, we compared the weight of those organs between VEGFA-DGranG and control females. There were no significant differences on the weight of uterus (0.1094 ± 0.0094

VS 0.1036 ± 0.0103 g, $P = 0.68$, fig. 2B), kidneys (0.3782 ± 0.0157 VS 0.4231 ± 0.0208 g, $P = 0.13$, fig. 2C) or adrenals (0.0082 ± 0.0007 VS 0.0087 ± 0.0005 g, $P = 0.54$, fig. 2D) between VEGFA-DGranG mice and control mice. So only ovarian weight was affected by granulosa cell specific VEGFA loss.

Effect of Granulosa cell-specific VEGFA loss on 17 beta estradiol, progesterone, and testosterone concentrations:

Blood collected was used to measure circulating hormone levels. Serum 17 β -estradiol concentrations in the VEGFA-DGranG females were 55% of those in controls (48.83 ± 9.58 VS 89.48 ± 13.17 pg/ml, $P < 0.05$, fig. 4A). There was no significant difference in progesterone concentrations between the VEGFA-DGranG and control females (145.49 ± 31.81 VS 135.39 ± 15.66 ng/ml, $P = 0.78$, fig 4B). Serum testosterone was not significantly different between the VEGFA-DGranG mice and control mice (0.12 ± 0.02 VS 0.16 ± 0.02 ng/ml, $P = 0.26$, fig 4C).

Effect of Granulosa cell-specific VEGFA loss on gene expression in ovary:

Because one study demonstrated that injection of a VEGFA inhibitor to prepubertal eCG treated rats significantly increased the levels of Bax and decreased the levels of Bcl-2 protein content of antral follicle (Abramovich *et al.*, 2006). We evaluated the effect of *Bcl-2* and *Bax* mRNA expression in Granulosa cell VEGFA loss within the ovary. *Bcl-2* and *Bax* mRNA in VEGFA-DGranG were not significant different from control (*Bcl-2*: 6.51 ± 2.27 VS 11.07 ± 4.50 , $P = 0.53$, fig. 5A; *Bax*: 5.76 ± 2.57 VS 4.41 ± 1.10 , $P = 0.56$, fig. 5B). In addition, there was no difference in ratio of *Bcl-2* to *Bax* (3.36 ± 2.60 VS

87.84 \pm 73.10, $P = 0.28$, fig. 5C). But *Bcl-2* averages were 2 fold higher in controls versus VEGFA-DGranG KO's and the ratios of *Bcl-2* to *Bax* was 26 fold higher in controls. Thus, variation in our control's severely affected our ability to detect differences in this gene and gene ratio. Forkhead boxo3a (*Foxo3a*) is a transcription factor and arrests follicle progression. *Foxo3a* mRNA was not significantly different between VEGFA-DGranG ovary and control (8.80 \pm 3.62 VS 4.99 \pm 1.24, $P = 0.23$, fig. 5D). Nor was an oocyte marker, growth differentiation factor (*Gdf-9*) (Sun *et al.*, 2010) mRNA different between VEGFA-DGranG ovary and control (148.91 \pm 59.89 VS 223.45 \pm 70.99, $P = 0.53$, fig. 5E). *Cyp11a1* converts cholesterol to pregnenolone and *Cyp17a1* converts pregnenolone to androgen. As shown in figure 5F and 5G, *Cyp17a1* (3851.28 \pm 3079.76 VS 1340.91 \pm 436.37, $P = 0.48$) and *Cyp11a1* (82,659.33 \pm 37,082.03 VS 8,509,574.76 \pm 8,420,312.39, $P = 0.48$) mRNA in the ovary between VEGFA-DGranG and control was not statistically significant different. *Cyp11a1* mRNA in VEGFA-DGranG ovary is 103 fold higher in controls; but the variation in control's *Cyp11a1* gene expression and reduced numbers of KOs inhibited our ability to detect differences in this gene.

Effect of Granulosa cell-specific VEGFA loss on fertility:

To determine whether Granulosa cell-specific VEGFA loss has any effect on fertility, VEGFA-DGranG females were mated with males that had VEGFA knocked out in Sertoli and some germ cells (VEGFA-DSertG). There were 5 KO groups and 9 control groups. We found that the number of days for VEGFA-DGranG females mating with VEGFA-DSertG males until parturition was (12 days) longer than that for control

females mating with control males (38.27 ± 4.65 VS 26.18 ± 2.17 days, $P < 0.05$, fig. 6). This indicated that the rate of ovulation in female mice or sperm quantity in the males may be affected by VEGFA-specific granulosa and Sertoli knock outs. There was no significant difference in number of litters/female in approximate 2 months, pups/litter between VEGFA-DGranG females with VEGFA-DSertG males group and control females with control males group (2.20 ± 0.37 VS 1.67 ± 0.24 litter numbers/female in approximate 2 months, $P = 0.23$; 8.18 ± 0.74 VS 7.64 ± 0.40 pups/litter, $P = 0.50$).

Discussion

In the current study, we determined that VEGFA loss in granulosa cells increased the time from mating to parturition, reduced number of antral follicles, numbers of CL/ovary and reduced estrogen concentrations by 55%. Thus, these females were subfertile due to the loss of both angiogenic and anti-angiogenic VEGFA isoforms. The reduction in fertility may be due to less overall follicle progression and maturation, reduced ovulations, or through reduced production of estrogen. The reduction in follicle maturation is supported by the smaller ovarian weight and reduction in estrogen concentrations in blood; suggesting that less follicles reached the antral stage where estrogen production is maximal.

The formation of a dense capillary network (angiogenesis) in the ovary enables the hormone-producing cells to obtain the oxygen, nutrients and also precursors necessary to synthesize and release different hormones essential for maintenance of the ovarian functions (Kaczmarek *et al.*, 2005). Loss of VEGFA in granulosa cells may reduce

angiogenesis, the blood supply for oxygen, nutrients and influence the growth of the ovary and follicle maturation. The reduction in ovarian weight in the VEGFA-DGrnG females may be due to a decline in maturation of follicles and this is also supported by the dramatic reduction in estrogen that was present in these females.

Follicles are surrounded by somatic cells (granulosa and theca cells) and can be classified into preantral (primordial, primary and secondary) and antral (tertiary and preovulatory) follicles. Quiescent primordial follicles and slow growing preantral follicles do not have a vascular supply of their own, but instead rely on vessels in the surrounding stroma (Brunol *et al.*, 2009). Stromal blood vessels transport nutrients and oxygen to primordial and primary follicles through passive diffusion. Follicular growth is associated with the development of an individual capillary network and continued angiogenesis to nourish the rapidly expanding follicle. Some types of infertility may be associated with the disturbance of follicular angiogenesis, which results in inadequate development (Fraser, 2006). Acquisition of an adequate vascular supply is probably a limiting step in the selection and maturation of the dominant follicle destined to ovulate (Stouffer *et al.*, 2001).

Our lab also demonstrated that VEGFA is involved in primordial follicle activation, maturation and survival (McFee *et al.*, 2009). Rat ovaries treated with VEGFA₁₆₄ or VEGFA_{xxx}B antibody had a 93% increase in vascular density. VEGFA₁₆₄ treated ovaries were composed of fewer primordial follicles (stage 0) and more developing follicles (stages 1–4) than controls. Ovaries treated with VEGFA_{xxx}B antibody had fewer primordial and early primary follicles and more primary, transitional, and secondary

follicles compared to controls. These data suggested that VEGFA proangiogenic isoform promote follicle development, and in contrast, VEGFA anti-angiogenic isoforms maintain primordial follicles in an arrested state. Removal of VEGF anti-angiogenic isoforms allows for increased progression of primordial follicles to the developing follicle pool (Artac et al., 2009). VEGFA also promotes the primary into secondary follicle transition in bovine follicles activated *in vitro* stimulating follicular growth in cattle (Yang and Fortune *et al.*, 2007) as well as rodents.

In our study, ovarian weight in VEGFA-DGranG mice was smaller than that of control. This suggests granulosa cell-specific VEGFA loss within mouse ovary does affect ovarian size through reduction of follicle development and/or maturation. In previous studies direct injection of VEGFA in the rat ovary increased the number of antral follicles and promoted the development of antral follicles by inducing the formation of theca vasculature. The results of VEGFA treatment were increased cross-sectional area of ovaries with VEGFA injection compared to controls (Shimizu *et al.*, 2007). Another reason for the reduced ovarian size in the VEGFA-DGranG females is due to the reduced CL and potentially a decrease in ovulation rate. An *in vivo* study showed that the VEGFA angiogenic isoform promoted ovarian follicular angiogenesis leading to stimulatory effects on subsequent follicular development and numbers of oocytes ovulated in mature cycling rats (Iijima *et al.*, 2005). Inhibition of VEGFA and KDR suppress follicular development and ovulation (Zimmermann *et al.*, 2001; Zimmermann *et al.*, 2002). The fewer CL in VEGFA-DGranG may be caused by fewer

antral follicles and less ovulation in those mice. Even though CL numbers were reduced there was no significant difference in progesterone concentrations in the serum.

Our previous data showed that rat ovaries treated with VEGFA 164 or VEGFAxxxB antibody increased the vascular density (Artac *et al.*, 2009). In newly formed CL, VEGFA mRNA and protein expression are observed in the granulosa- and theca-derived luteal cells. In several species, expression levels are higher in granulosa-derived cells than that in theca-derived luteal cells (Kamat *et al.*, 1995; Endo *et al.*, 2001; Boonyaparakob *et al.*, 2003; Kaczmarek *et al.*, 2005). Highly expressed VEGFA in granulosa-derived luteal cells may act as a chemoattractant for endothelial cells in order to initiate the invasion of avascular granulosa layer establishing an extensive capillary network that nourishes the development of CL and assists in the maintenance of luteal function throughout its' lifespan (Kaczmarek *et al.*, 2005). In the present study, two ovaries of VEGFA-DGranG mice showed abnormal morphological structure in CL which appeared to be devoid of vasculature. It is possible that granulosa cell-specific VEGFA loss affects the establishment of capillary network in developing CL and consequently influences the structure of CL.

Estrogens are produced primarily by developing follicles in the ovary (Yang *et al.*, 2000). Other tissues such as the liver, adrenal glands, and the breast also produce estrogens in a smaller amount. Granulosa cells in the ovary can convert androstenedione to estrone or estradiol. The aromatase knockout mouse, the estrogen β receptor knockout mouse, and the estrogen α receptor knockout mouse have demonstrated that estrogen enhances the number of follicles progressing to the early antral stage (Fisher *et al.*, 1998;

Krege *et al.*, 1998; Schomberg *et al.*, 1999; Danforth *et al.*, 2003). Furthermore, a positive relationship between VEGFA and E₂ concentrations in the gilt follicular fluid suggest a possible cause-and-effect relationship (Mattioli *et al.*, 2001) with VEGF stimulating estrogen production by increasing vascular permeability to cholesterol and increasing signal transduction pathways activating enzymes that are critical to estrogen production. One study showed that serum 17 β -estradiol concentration in rats treated with VEGFA120 and 164 were significantly higher than that in the control rats, indicating that VEGFA120 and 164 may indirectly stimulate the production of 17 β -estradiol via granulosa cells (Iijima *et al.*, 2005). In our study, serum 17 β -estradiol level in the VEGFA-DGranG was significantly lower than that in the control. Our results indicated that loss of VEGFA in granulosa cell may inhibit the production of 17 β -estradiol via granulosa cells in the mouse.

VEGFA suppresses apoptosis in vascular endothelial cells by regulating Bcl-2 family proteins (Gerber *et al.*, 1998; Nör *et al.*, 1999). The members of this family consist of anti-apoptotic factors (such as Bcl-2 and Bcl-xL) and pro-apoptotic factors (such as Bax). Bcl-2 maintains permeability of the mitochondrial membrane. However, Bax increases permeability of the mitochondrial membrane and stimulates apoptosis inducers (such as cytochrome C and Smac/Diablo) which were released from mitochondria into the cytosol. The mitochondrial apoptotic inducers in the cytosol stimulate the activation of the caspase cascade. Activated caspase cascades can result in apoptosis. VEGFA is a key survival factor for endothelial cells and induces the expression of Bcl-2 in human endothelial cell (Gerber *et al.*, 1998). Local inhibition of VEGFA activity appeared to

produce an increase in ovarian apoptosis through an imbalance among the follicular contents of Bcl-2 family members' protein, thus leading a larger number of atretic follicles (Abramovich *et al.*, 2006).

However, VEGFA does not stimulate or inhibit Bcl-xL and Bax, respectively, in bovine Granulosa cells. It is hypothesized that VEGFA does not suppress the expression of active caspase-3. VEGFA suppresses granulosa cell apoptosis by suppressing the downstream activities of caspase-3 without being associated with the mitochondrial pathway. Because VEGFA has tyrosine kinase receptors, it may act via Akt or Erk in the downstream cascade of caspase-3 to inhibit granulosa cell apoptosis (Kosaka *et al.*, 2007). The exact mechanism of how VEGFA acts on the granulosa cell or follicular survival still needs to be elucidated. Besides *Bcl-2* and *Bax*, we also determined expression of other genes in the ovary.

Foxo3a is a key oocyte factor critical for suppressing primordial follicle activation (Castrillon *et al.*, 2009). *Foxo3a* mRNA is 1.76 fold higher in KO's ovary than control. Although, the difference is not significant, the higher value in KO may suggest follicle progression is suppressed in these ovaries. Gdf-9, produced by oocyte, plays critical roles in early follicular development. This oocyte-derived paracrine factor also stimulates follicular somatic cell proliferation and steroidogenesis (Elvin *et al.*, 1999). There is no statistical significant difference on *Gdf-9* mRNA between the KO's ovary and controls. However, the mRNA average is 34% lower in VEGFA-DGranG ovary. Thus, less *Gdf-9* expression may inhibit follicular development, somatic cell proliferation and estrogen synthesis. We did not have enough tissue to evaluate protein for Bcl-2, Bax, Foxo3a, or

Gdf-9. Thus it is also possible that difference in protein production of these genes result from VEGFA isoform loss.

Our KO mice from the present study were subfertile. The time period from mating to parturition in female VEGFA-DGranG mice was 12 days longer compared to the control. This suggested a decreased rate of ovulation in the female VEGFA-DGranG mice or ovulation of oocytes that are not competent to ovulate. Ha *et al.*, suggest that estrogen administration improves number and quality of oocytes which may possibly be mediated by the increase of ovarian VEGF expression. In our study, reduced estrogen may reduce the number and quality of oocytes and VEGFA expression in the ovary. Furthermore, VEGFA loss in granulosa cells may diminish the number of healthy preovulatory follicles and ovulated oocytes.

Follicular vascularization appears to have a critical influence on the development of competing human oocytes and early embryos. Follicular vascularity and oxygen content appear to be important factors of oocyte competence (Van. 1998). In an *in vivo* experiment, Iijima *et al.* treated mature female rats with VEGFA120 or 164 and an angiogenic inhibitor (TNP-470) during the estrous cycle. They reported that VEGFA angiogenic treatment stimulated follicular angiogenesis, while TNP-470 treatment showed severe depression of follicular angiogenesis and completely inhibited ovulation. After treatment with VEGFA 120 and 164, the number of healthy preovulatory follicles and ovulated oocytes significantly increased and the number of atretic preovulatory follicles decreased (Iijima *et al.*, 2005). In Iijima's experiment, the oocytes ovulated had a normal ability to fertilize and develop to term with the same litter size as in the control

rats. In the current study, to determine the quality of the oocytes ovulated by granulosa cell-specific VEGFA loss, VEGFA-DGranG mice were mated with VEGFA-DSertG mice and allowed to proceed to pregnancy. The number of litters/female in approximate two months or pups/litter was not significantly different between the VEGFA-specific knockout mice and controls. However the time to produce a litter was longer in our knockout matings suggesting that there may be multiple problems in obtaining viable embryos that are capable of being maintained for a complete pregnancy. Thus, granulosa cell-specific VEGFA loss may have effects on embryo viability.

In conclusion, we demonstrated that removal of VEGFA in granulosa cells within ovaries resulted in reduced ovarian weight, fewer CL and reduced estrogen concentrations (by 55%). Furthermore, the time period between mating and parturition is longer in female VEGFA-DGranG KO's than that in controls. The present data suggest that VEGFA is necessary for normal follicular development, ovulation rate, estrogen concentration and timing of mating to parturition and without VEGFA angiogenic and antiangiogenic isoforms females are subfertile.

Figure Legends

Table 1. Quantitative Real-time PCR primers, probes and kit.

Figure 1. Effect of granulosa cell-specific VEGFA loss on morphology of ovary. Histological images of sections (H&E stained) of control ovaries (A, C, E) and VEGFA-DGranG ovaries (B, D, F, G). Two VEGFA-DGran ovaries had abnormal morphology in structures that appeared to be CL (F, G).

Figure 2. Effect of granulosa cell-specific VEGFA loss on ovaries (A) uterus (B), kidneys (C) and adrenals (D) in female mouse. The mean weights \pm SEM are presented. * denotes significant difference at $P < 0.05$.

Figure 3. Cyp11a1 immunohistochemistry (IHC) images (A, B, C) and effect of granulosa cell-specific VEGFA loss on the numbers of Corpus Lutea (D). A is showing the H&E stained CL, B is showing Cyp11a1 IHC stained CL (brown color), and C is the negative control for IHC. The mean numbers of CL per ovary \pm SEM are presented. * denotes significant difference at $P < 0.05$.

Figure 4. Effect of granulosa cell-specific VEGFA loss on the serum E₂ (A), progesterone (B) and testosterone (C) concentrations. The mean serum concentrations \pm SEM are presented. * denotes significant difference at $P < 0.05$.

Figure 5. Effect of granulosa cell-specific VEGFA loss on *Bcl-2* (A), *Bax* (B), *Bcl-2/Bax* (C), *Foxo3a* (D), *Gdf-9* (E), *Cyp17a1* (F) and *Cyp11a1* (G) mRNA expression in the ovary. The mean normalized values \pm SEM are presented.

Figure 6. Time from mating control female with control male to parturition VS time from mating VEGFA-DGranG females with VEGFA-DSertG males to parturition. There were 5 KO groups and 9 control groups. The mean days from mating to parturition \pm SEM are presented. * denotes significant difference at $P < 0.05$.

Gene	Forward Primer	Probe Dye	Probe Vendor	Reverse Primer
<i>Gapdh</i>	Kit-Taqman Rodent GapDH Control Reagents Applied Biosystems Catalog #4308313 (VIC Probe)			
<i>Bcl-2</i>	CGTTGGCCCTTCGGAGTT	FAM	Applied Biosystems	TCAGACCTCCAGGGCAGAGA
<i>Bax</i>	GGCCTTTTGTCTACAGGGT TT	FAM	Applied Biosystems	GTGTCTCCCCAGCCATCCT
<i>Foxo3a</i>	GCAAAGCAGACCCTCAAA CTG	SYBR Green	Applied Biosystems	TGAGAGCAGATTTGGCAAAGG
<i>Gdf-9</i>	GCCGGGCAAGTACAGCC	SYBR Green	Applied Biosystems	TTTGTAAAGCGATGGAGCCG
<i>Cyp17a1</i>	TGGAAGGATGCACAGGTT GA	SYBR Green	Applied Biosystems	ATGGGATCCGGGACGTTAG
<i>Cyp11a1</i>	CCCCCTGGGTGGCCTAT	SYBR Green	Applied Biosystems	TGAACTCTTAAACAGGACCCCA AT

Table 1.

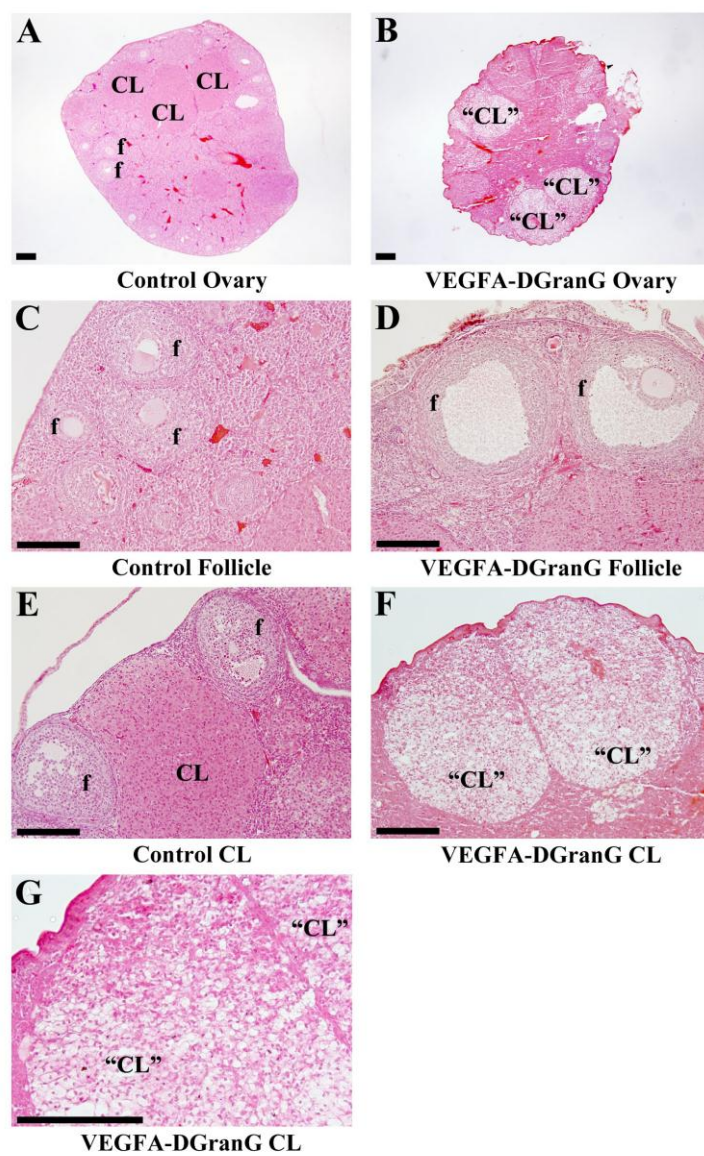


Figure 1.

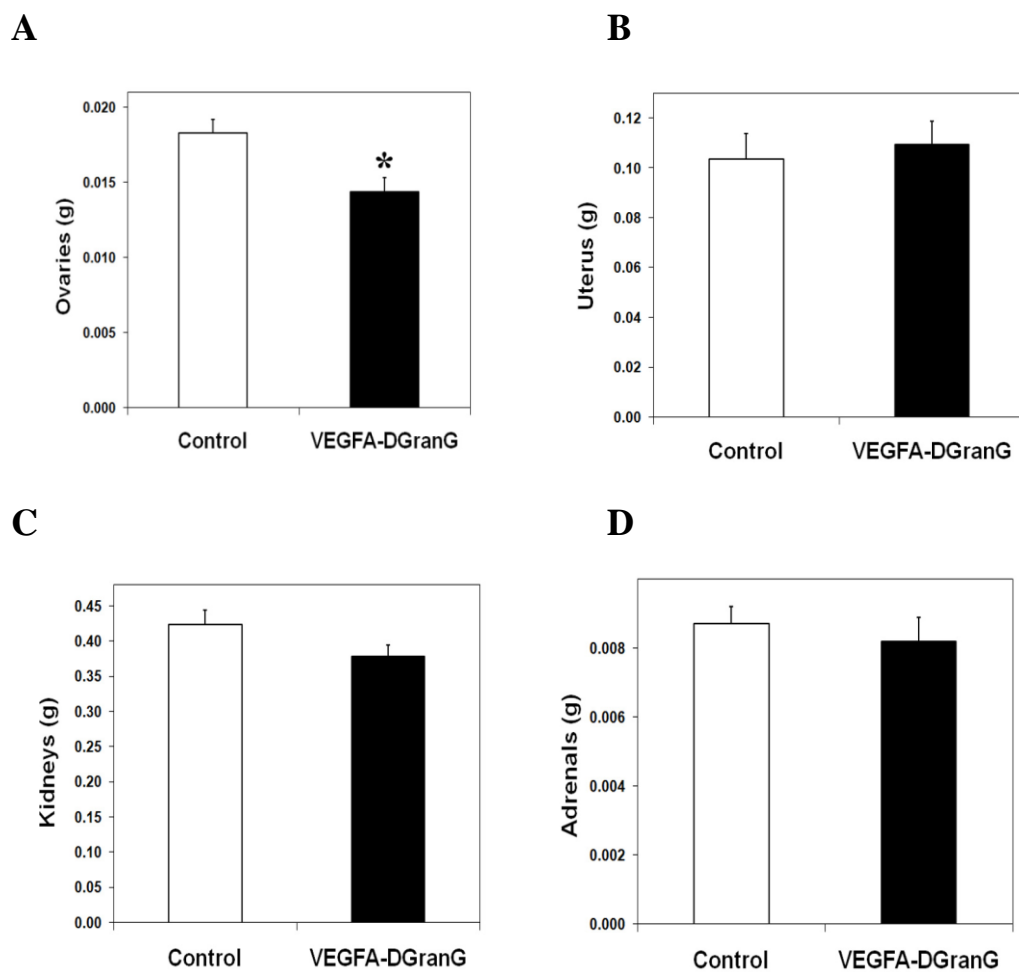
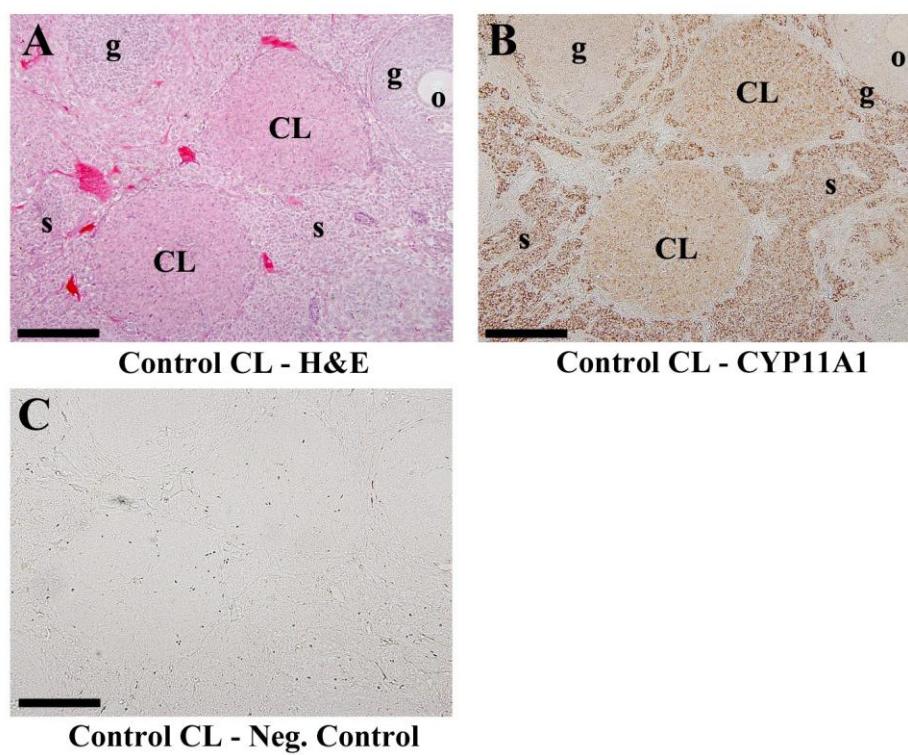
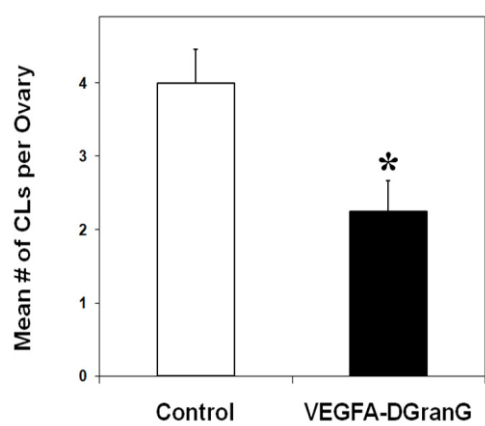


Figure 2.

**D****Figure 3.**

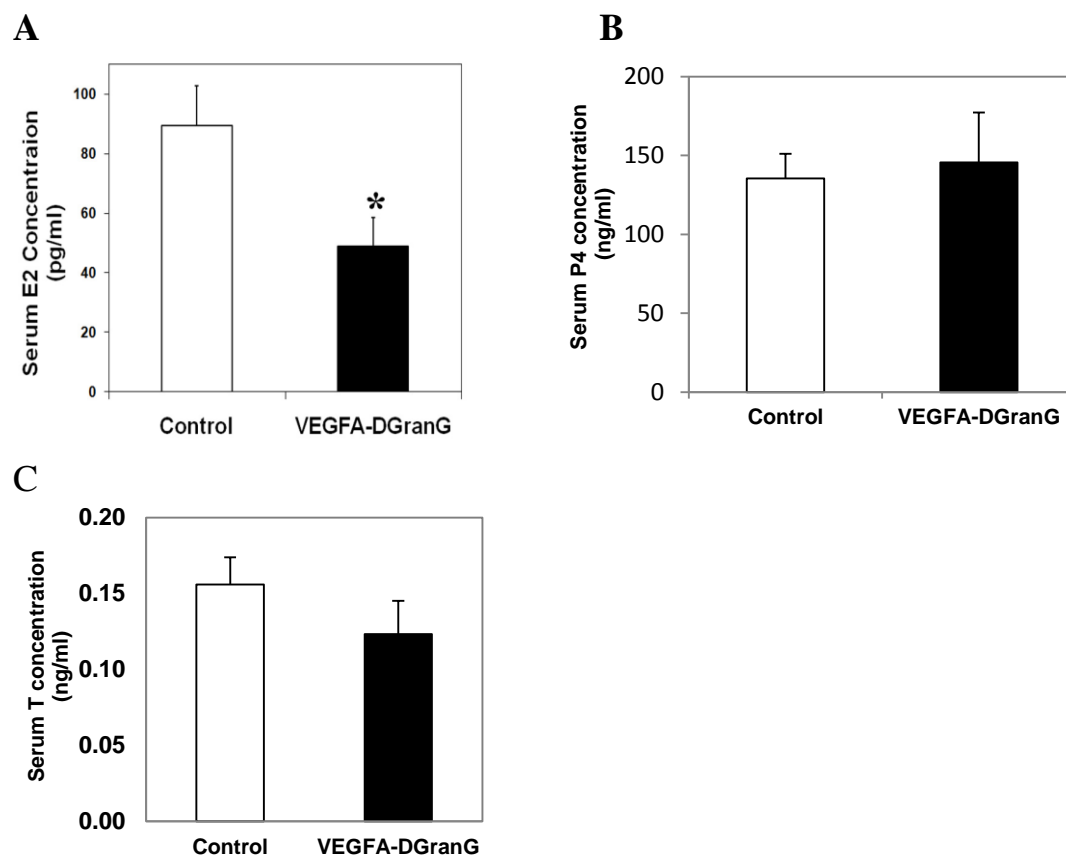
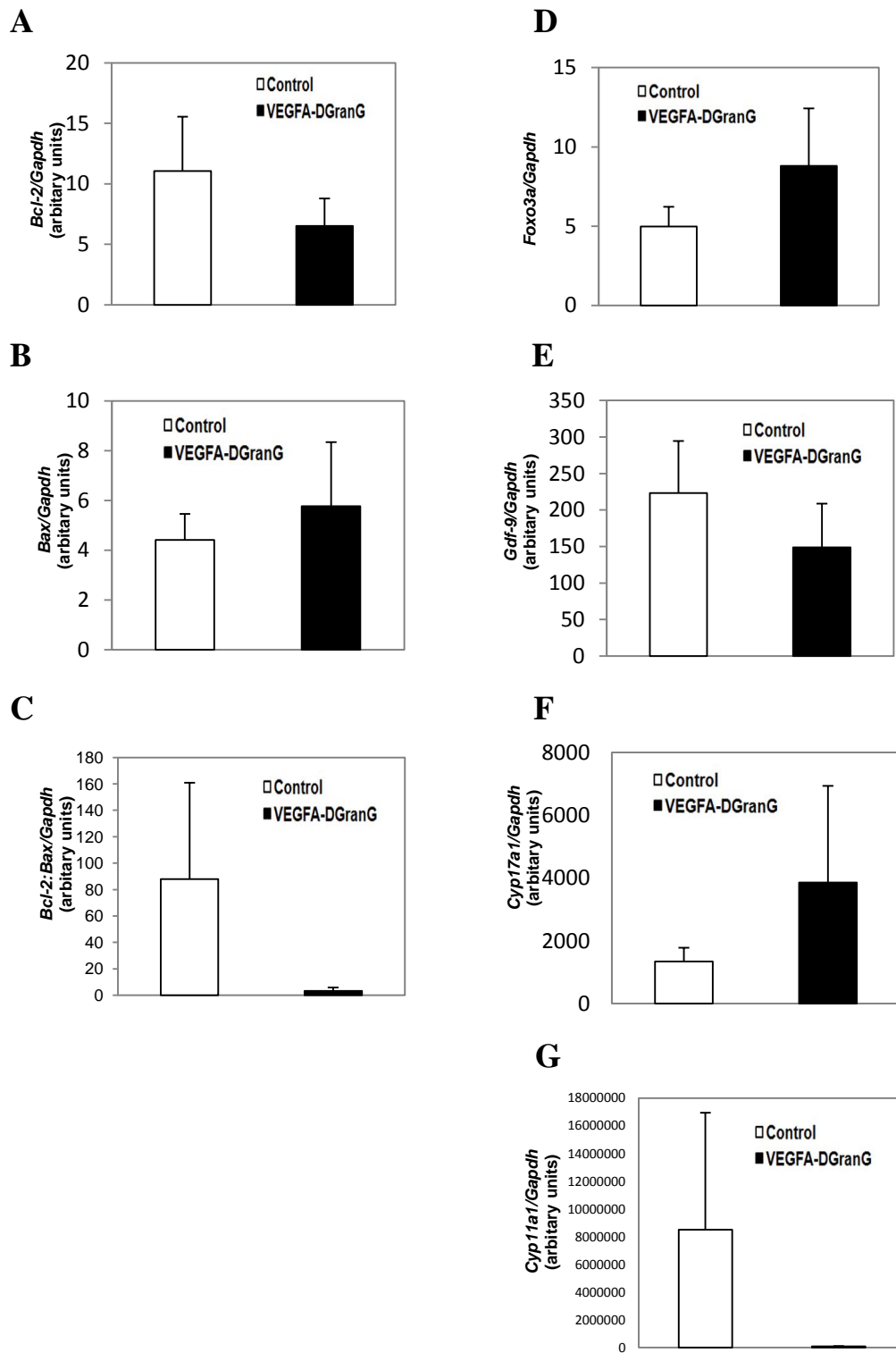


Figure 4.

**Figure 5.**

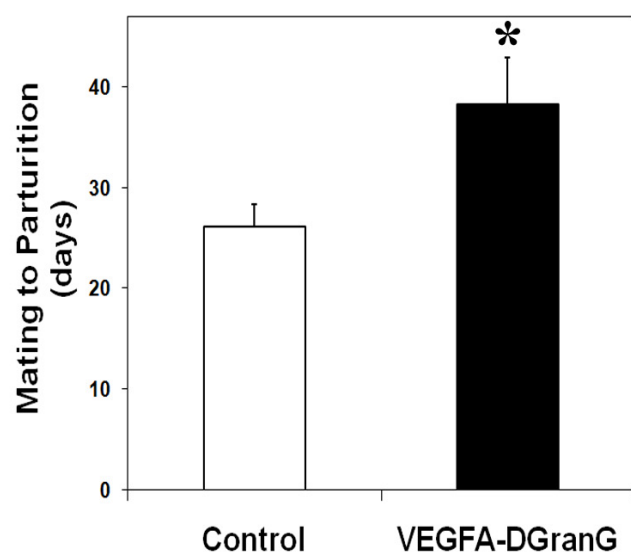


Figure 6.

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Chapter 3

Sertoli cell-specific Vascular Endothelial Growth Factor A (VEGFA) loss using pDMRT-1 Cre reduces testis size, increases testosterone, and affects genes involved in the apoptotic pathway resulting in subfertile males

Abstract:

VEGFA is present during testis morphogenesis and is expressed by Sertoli cells and germ cells. Inhibition of VEGFA disrupts both testis-specific vascular development and cord formation. We hypothesize that production of VEGFA isoforms by Sertoli cells is critical for normal testis morphogenesis. Therefore, the objective of the current study was to evaluate the effects of Sertoli cell-specific production of VEGFA isoforms on sex-specific vascular development and gonadal morphogenesis. The *pDmrt1* gene is expressed in the indifferent gonad in precursor Sertoli and granulosa cells and in some sub-populations of germ cells. Therefore, we used a pDMRT1-cre crossed to a floxed VEGFA mouse to generate male Sertoli-cell (VEGFA-DSertG) specific knockouts. Reproductive organs were collected from adult mice, and weighed (control n=9, VEGFA-DSertG n=8). Testosterone concentrations in blood were determined by ELISA. In VEGFA-DSertG males, the body weight (28.52 ± 0.48 VS 32.52 ± 1.20 g); testes (0.1658 ± 0.0064 VS 0.1924 ± 0.0061 g) and epididymal (0.0627 ± 0.0046 VS 0.0792 ± 0.0042 g) weight were significantly reduced compared to controls ($P < 0.05$). The prostate weight in VEGFA-DSertG males also tended to be smaller than controls (0.0104 ± 0.0031 VS 0.0215 ± 0.0044 g, $P = 0.06$). Morphologically the epididymis and

prostate were different with fewer tubules and sperm in the epididymis and reduced branching in the prostate compared to controls. Serum testosterone levels in VEGFA-DSertG males tended to be higher than those in controls (5.28 ± 2.24 VS 0.97 ± 0.33 ng/ml, $P = 0.097$). Three VEGFA-DSertG males had 10 fold greater testosterone concentrations than controls (12.81 VS 0.97 ng/ml). Relative amounts of *Bcl-2* mRNA in VEGFA-DSertG testes tended to be greater than that in controls (4.51 ± 1.26 VS 1.89 ± 0.27 , $P = 0.07$). The ratio of *Bcl-2/Bax* tended to be increased in VEGFA-DSertG testes compared to control (1.20 ± 0.33 VS 0.54 ± 0.08 , $P = 0.08$). Relative amounts of *Bax* (3.71 ± 0.52 VS 4.10 ± 0.53 , $P = 0.67$), *Sin3a* (34.17 ± 9.97 VS 18.72 ± 2.90 , $P = 0.17$) and *aromatase* (10.77 ± 2.3 VS 9.01 ± 1.77 , $P = 0.56$) mRNA in VEGFA-DSertG testes were not significantly different compared to control. But *Sin3a* mRNA in three VEGFA-DSertG males with high testosterone concentration was significantly higher than controls (42.08 ± 13.91 VS 18.72 ± 2.90 , $P = 0.01$). Therefore, we can conclude from these data that lack of VEGFA in Sertoli cells (and sub-populations of germ cells) reduces gonadal and male accessory gland organ weights, alters steroidogenic profiles and *Bcl-2* and *Sin3a* expression in testis. Thus, VEGFA plays an important role in male reproductive organ morphogenesis and function in mice.

Introduction

Human fertility rates have declined all over the world. Currently, increased human infertility suggests that significant reproductive health problems exist (Skakkebaek *et al.*, 2005) and this is why they have identified a new disorder- testicular dysgenesis syndrome (TDS). Male reproductive health disorders, including impaired semen quality, testicular

cancer, cryptorchidism and hypospadias are all associated with testicular dysgenesis syndrome which is thought to occur due to abnormal embryonic or fetal development of cells involved in testis differentiation and morphogenesis (Joensen *et al.*, 2009).

Testis development is initiated by Sertoli cell differentiation and expression of Sry, causing mesonephric cell migration into the differentiating testis to form seminiferous cords (Cupp and Skinner 2005). Seminiferous cord formation and sex-specific vascular formation are only present in the testis and therefore are first to distinguish morphology in the indifferent gonad. Vascular endothelial growth factor A (VEGFA) is a paracrine growth factor responsible for blood vessel development as well as endothelial cell migration. Expression of VEGFA in normal testes, prostate and seminal vesicles and semen suggests that VEGFA could play a role in male reproductive physiology (Huminiecki *et al.*, 2001; Obermair *et al.*, 1999).

The *Vegf* family includes *Vegfa*, *Vegfb*, *Vegfc*, *Vegfd* (officially called *Figf*, c-fos-induced growth factor) and placenta growth factor (*Pgf*). The predominant gene in *Vegf* family is *Vegfa*. The *Vegfa* gene is composed of eight exons and by different factors produces angiogenic or inhibitory isoforms. These two isoforms have opposite functions (angiogenic isoforms promote vasculature development while anti-angiogenic isoforms inhibit vascular development). Two related tyrosine kinases receptors, FMS-like tyrosine kinase 1 (FLT1, also known as VEGFR1) and kinase insertion domain receptor (KDR, also known as VEGFR2 and FLK-1), bind to VEGFA. The primary receptor involved in the action of VEGFA is KDR (Ferrara *et al.*, 2003). KDR is the major mediator of the mitogenic, angiogenic and permeability effects of VEGFA. It has been demonstrated that

KDR participate in mediating endothelial cell proliferation, survival and vascular permeability, whereas FLT1 might play an inhibitory role by sequestering VEGFA, and preventing its interaction with KDR (Ferrara *et al.*, 2004).

VEGFA and its receptor KDR are detected at the time of testis morphogenesis. VEGFA is expressed by Sertoli cells and other cells within the seminiferous cords in early development (Bott *et al.*, 2006). FLT1 and KDR were expressed by both Leydig cells and germ cell of the testis (Korpelainen *et al.*, 1998; Bott *et al.*, 2006; Bott *et al.*, 2010). It has been demonstrated that overexpression of VEGFA in the testis and epididymis of transgenic mice leads to upregulation of VEGFA receptors in the endothelial cells, in certain spermatogenic cells and the Leydig cells, and causes infertility. The VEGFA overexpression in testis and epididymis exhibited spermatogenic arrest and the ductus epididymidis was dilated, containing areas of epithelial hyperplasia in mice (Korpelainen *et al.*, 1998). In the previous studies, our laboratory demonstrated that VEGFA, a Sertoli cell secreted growth factor, is critical for both vascular development and cord formation. Treatment with VEGFA antagonists or VEGFA receptor signal transduction inhibitors arrested cord formation and reduced vascular density in organ cultures of rat testes (Bott *et al.* 2006). Treatment of E13 cultured mouse testes with VEGFA angiogenic isoform or an antibody to antiangiogenic isoforms (anti-VEGFA_{xxx}B) resulted in undefined and poorly developed seminiferous cords compared with the cords in control. Moreover, the vasculature density increased in the cultured mouse testes with VEGFA angiogenic isoform or an anti-VEGFA_{xxx}B. The results suggest that both pro- and antiangiogenic VEGFA isoforms are involved in the

development of vasculature and seminiferous cords in rat testes, and differential expression of these isoforms may be important for normal gonadal development (Baltes-Breitwisch *et al.*, 2010). Recently, we also found that KDR-LacZ testis cultured with VEGF receptor tyrosine kinase inhibitor (VEGFR-TKI) present reduced endothelial cell migration during testis development (Bott *et al.*, 2010). Taken together, these studies suggest that VEGFA, secreted by Sertoli cells, is involved in the development of vasculature and seminiferous cords in rodent testes.

We proposed that VEGFA plays an important role in testis morphogenesis and function. To investigate the effects of VEGFA *in vivo*, we generated Sertoli cell-specific VEGFA loss transgenic mice. The objective of present study is to determine the *in vivo* effects of Sertoli cell-specific VEGFA loss on male reproductive organs' function and morphogenesis.

Materials and methods

Animals:

Sertoli cell-specific VEGFA KO mice were obtained by using the Cre-loxP approach. We used a line of VEGFA^{loxP/loxP} mice (Gerber *et al.*, 1999) from Napoleon Ferrara at Genentech to mate with pig DMRT1-cre mice (Boyer *et al.*, 2002) to develop Sertoli and granulosa cell-specific VEGFA knockout mice (breeding scheme in Appendix A). The *Dmrt1* gene is expressed in the indifferent mouse gonad at 10.5 dpc in precursor cells that differentiate into Sertoli and granulosa cells. By 12.5 dpc, *Dmrt1* is expressed in Sertoli cells and germ cells within XY gonads and in ovarian somatic cells and germ cells.

DMRT1 is presented in Sertoli cells at all ages examined and absent in ovarian somatic cells from 13.5 dpc. Expression of *Dmrt1* in primordial germ cells increases through 13.5 dpc and is absent by 15.5 dpc. In germ cell, DMRT1 was detected only in spermatogonia of the testis after birth (Lei *et al.*, 2007). The p*Dmrt1* gene is expressed similar to mouse *Dmrt1* (Boyer *et al.*, 2002). In our VEGFA-DSertG mouse, VEGFA gene exon 3 was flanked by two loxp and Cre, driven by pig *Dmrt1*, bound and deleted the DNA sequence between the two loxp. The VEGFA was not expressed in the cells where *Dmrt1* is present. Therefore, we expected potential phenotypes in male Sertoli and female granulosa cell (and sub-populations of germ cell) knockouts. We used Cre-negative VEGFA^{loxp/loxp} or VEGFA^{loxp/+} mice as controls and Cre-positive VEGFA^{loxp/loxp} mice as KO (VEGFA-DSertG). Testes, epididymis, prostate, seminal vesicles, kidneys and adrenals were collected from male adult mice (control n=9, VEGFA-DSertG n=8) and weighed. The average age for the KO group was 178 days and for the control group was 175 days. All animal protocols were approved by the University of Nebraska Institute of Animal Care and Use Committee (IACUC) in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Genotyping:

DNA was extracted from tail samples and used for genotyping via polymerase chain reaction (primers and PCR programs are listed in Appendix B). Genomic DNA was extracted from tails of mice by proteinase K digestion and followed by 6M NaCl extraction and ethanol precipitation (Bott *et al.*, 2010).

Embedding, Histology and staining (hemotoxylin and eosin staining; H&E):

Testes, epididymis and prostate were fixed in Bouin's solution and rinsed in 70% ethanol. Embedding was performed according to standard procedures (McFee *et al.*, 2009; Bott *et al.*, 2010). Prior to paraffin embedding, tissues were rinsed in 100% ethanol 1 hour for 3 times and CitriSolve (Fisher Scientific, Fairlawn, IL) 1 hour for 3 times. Embedded tissues were sectioned (5 μ m) and the slides were placed in an incubator (37 °C) overnight. Sectioned tissues were deparaffinized, stained with hematoxylin for 1 min (VWR International, West Chester, PA) and eosin for 30 min (Ricca, Arlington, TX), dehydrated and mounted.

Immunohistochemistry:

Sectioned tissues were deparaffinized, rehydrated, and microwaved in 0.01 M sodium citrate to boil for 15 min. Then, tissues were cooled for 1 h and rinsed in 3% hydrogen peroxide in methanol for 20 min. Sections were blocked with 10% normal goat serum in PBS for 30 min at room temperature and washed in PBS 3 times. Immunohistochemistry was performed according to the standard protocol in our laboratory (Mcfee *et al.*, 2009). DDX4/MVH, a primordial germ cell marker, (catalog number: ab13840; Abcam, Cambridge, Mass., USA) was diluted 1:500 in 10% normal goat serum. The aromatase primary antibody (catalog number: ab18995; Abcam) was diluted in 1:100 in 10% normal goat serum. As a negative control, serial sections were processed without primary antibody. Biotinylated goat anti-rabbit secondary antibodies

were diluted 1:300. Secondary antibody was detected using aminoethyl carbazole (AEC) chromagen substrate solution (ZYMED Laboratories, San Francisco, CA).

RNA isolation:

Total RNA from testes was extracted using Tri-Reagent according to manufacturer's protocol (Sigma, St. Louis, MO). After isolation total RNA was dissolved in 20 μ l diethyl pyrocarbonate (DEPC) water. The extracted RNA was diluted 1:14 in DEPC water and the concentration was determined by spectroscopy at 260nm using the Lambda EZ 150 (Perkin Elmer).

Reverse Transcription (RT)-PCR:

Before RT-PCR, 1 μ l of RNA was removed from each sample to a new tube with 15 μ l DEPC water to be used as "No RT" to detect the residual genomic DNA. RT was performed on 5 μ g of template using SuperScript II (Invitrogen, Carlsbad, CA) according to the manufacturer's recommended protocol (McFee *et al.*, 2009; Bott *et al.*, 2010). RT samples were incubated in 65°C with 2 μ l of oligo (dT) 15 primer (Promega), 2 μ l dioxynucleotide triphosphates (Fermantas; 10 mM dNTP mix), 3.8 μ l DEPC water, and then immediately chilled on ice. After that, 1 μ l SuperScript II reverse transcriptase (200 units/ μ l; Invitrogen, Carlsbad, CA), 3.2 μ l of stand buffer (Invitrogen, Carlsbad, CA), 2 μ l 0.1 M DL-dithiothreitol (DTT) (Invitrogen, Carlsbad, CA), 1 μ l DEPC water were added into each sample. The samples were incubated at 42°C for 1 hour followed by 15 mins at 70°C to inactivate the reverse transcriptase. The cDNA was stored at 20°C for subsequent real time-PCR.

Quantitative RT- PCR (QRT-PCR):

Primers, probes and kit used in the experiment were shown in table 1. Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) gene is a housekeeping gene and used as a control for RNA amplification. QRT-PCR was performed in 384 well plates (Axygen Scientific, Union City, CA) with an adhesive cover film (VWR, Scientific Products, North Kansas City, MO). *Bcl-2*, *Bax* and *Aromatase* were performed with 10 μ l Taqman universal Master Mix (Applied Biosystem, Foster City, CA), 1 μ l cDNA, 0.5 μ l of each forward and reverse primers, 2 μ l probe, and 6 μ l DEPC to make up the reaction volume to 20 μ l. *Sin3a* were performed with 10 μ l Taqman universal Master Mix (Applied Biosystem, Foster City, CA), 1 μ l cDNA, 1 μ l of primers and probe mix (Applied Biosystem, Foster City, CA), and 8 μ l DEPC to make up the reaction volume to 20 μ l. Experimental and *Gapdh* PCRs were carried out in separate wells in triplicate. An arbitrary value of template was assigned to the highest standard and corresponding values were assigned to the subsequent dilutions. These relative values are plotted against the threshold value for each dilution to generate a standard curve. The relative amount for each experimental and *Gapdh* triplicate was assigned an arbitrary value based on the slope and y-intercept of the standard curve. The average of the experimental triplicate is divided by the average of the *Gapdh* triplicate, and the resulting normalized values are used for statistical analysis (McFee *et al.* 2009).

Blood collection and Hormone Enzyme-Linked Immunosorbent Assay (ELISA):

Blood was collected at the time of euthanization and put into tubes with 20 μ l EDTA solution (Fisher Scientific Co., Fair Lawn, NJ). Blood sample was immediately centrifuged at 1250 X g for 10 min; the plasma was removed into a new tube and stored in -80 °C. Testosterone concentration was determined by Testosterone ELISA kit (Alpha Diagnostics International Inc., San Antonio, TX; Cat. No.1880). Standard curve samples were made of 0 ng/ml, 0.5 ng/ml, 1 ng/ml, 2.5 ng/ml, 10 ng/ml and 20 ng/ml. Assay sensitivity was 0.125 ng/ml.

Statistical analysis:

All data were analyzed by two-sample *t*-test using SAS software (SAS institute, Cary, NC). Differences in data were considered statistically significant at $P < 0.05$

Results

Phenotypes in the male testis of VEGFA-DSertG mice:

In VEGFA-DSertG, the body and testes weights were significantly smaller than those from the control (body: 28.52 ± 0.48 VS 32.52 ± 1.20 g; testes: 0.1658 ± 0.0064 VS 0.1924 ± 0.0061 g, $P < 0.05$; fig. 1G and 1H). To assess the effect of Sertoli cell-specific VEGFA loss on testes histological morphology, testes were stained with H&E. There was no significant difference in morphology of testis (fig. 1A-F).

Phenotype of seminal vesicles, kidneys and adrenals in VEGFA-DSertG:

In VEGFA-DSertG, seminal vesicles, kidneys and adrenals weight were not significantly different compared to those in control (seminal vesicles: 0.2324 ± 0.0134 VS 0.2579 ± 0.0281 g, $P = 0.43$; kidneys: 0.4673 ± 0.0122 VS 0.4947 ± 0.0239 g, $P = 0.34$; adrenals: 0.0077 ± 0.0022 VS 0.0068 ± 0.0016 g, $P = 0.7$).

Gene expression in testis:

Because it is demonstrated that VEGFA angiogenic isoform treatment increased the expression of *Bcl-2* relative to *Bax* in bovine testis (Caires *et al.*, 2009). We evaluated *Bcl-2* and *Bax* mRNA expression in the testis. As shown in fig. 2A, *Bcl-2* in VEGFA-DSertG tended to be higher than that in control (4.51 ± 1.26 VS 1.89 ± 0.27 , $P = 0.07$). Sertoli cell-specific VEGFA loss stimulated production of *Bcl-2* mRNA in testis. *Bax* in VEGFA-DSertG was not different from controls (3.71 ± 0.52 VS 4.10 ± 0.53 , $P = 0.67$, fig. 2B). In addition, Sertoli cell-specific VEGFA loss tended to increase the ratio of *Bcl-2* to *Bax* (1.20 ± 0.33 VS 0.54 ± 0.08 , $P = 0.08$, fig. 2C). Swi-independent 3a (*Sin3a*) is specifically expressed in Sertoli cells in the testis and it is required by Sertoli cells to establish a niche for undifferentiated spermatogonia and spermatid elongation (Payne *et al.*, 2010). Relative amount of *Sin3a* mRNA was not significant different between VEGFA-DSertG testes and control (fig. 2D) (34.17 ± 9.97 VS 18.72 ± 2.90 , $P = 0.17$). However, the amount of *Sin3a* mRNA in those three male KOs with high testosterone was significantly higher than the controls (42.08 ± 13.91 VS 18.72 ± 2.90 , $P = 0.01$, fig. 2E).

Effect of Sertoli cell-specific (and sub-populations of germ cell) VEGFA loss on testosterone concentration in serum and aromatase in testis:

Blood was collected to test circulating hormone levels. Serum testosterone concentration in the VEGFA-DSertG mice tended to be higher than those in control mice (5.28 ± 2.24 VS 0.97 ± 0.33 ng/ml, $P = 0.097$, fig. 3G). The testosterone concentrations in three VEGFA-DSertG mice had 10 fold (13.83, 13.72, 10.88 ng/ml) higher concentrations compared to controls (0.97 ± 0.33 ng/ml, fig. 3H). Aromatase converts testosterone to estrogen in Sertoli cells. We stained for aromatase with immunohistochemistry. It is shown that aromatase was expressed in both Leydig cells, some germ cells and Sertoli cells. Aromatase positive staining in VEGFA-DSertG was not significantly different from that in the control (fig 3A-F). *Aromatase* mRNA (10.77 ± 2.3 VS 9.01 ± 1.77 , $P = 0.56$, fig. 3I) in testis was not significantly different between VEGFA-DSertG and controls.

Effect of Sertoli cell-specific (sub-populations of germ cells) VEGFA loss on germ cell:

To examine whether Sertoli cell-specific VEGFA loss alter the germ cell numbers in testis, we used immunohistochemistry for VASA (a known germ cell marker) to determine if there were differences in germ cell numbers. There was no difference in the germ cell number on randomly counted areas of the testis between the VEGFA-DSertG and control (fig. 4) as measured with VASA positive staining.

Effect of Sertoli cell-specific VEGFA loss on epididymis:

Epididymis is part of reproductive tract in the male, which was also observed in the current study. Epididymal weight in VEGFA-DSertG was significantly smaller than that in control (0.0627 ± 0.0046 VS 0.0792 ± 0.0042 g, $P < 0.05$, fig. 5G). To evaluate the morphology, the epididymis was embedded, sectioned and stained with hematoxylin and eosin (H&E). The epididymal head in VEGFA-DSertG appeared to have fewer tubules than the control. Besides the reduced numbers of tubules, VEGFA-DSertG epididymis also appeared to have fewer sperm in lumen than the controls (fig. 5A-F).

Effect of Sertoli cell-specific VEGFA loss on prostate:

The prostate is an important gland in males. The prostate gland produces, and secretes an alkaline fluid which is a part of semen and provides a protective buffer for sperm survival in the oviduct. In the present study, the prostate in VEGFA-DSertG males also tended to be smaller than controls (0.0104 ± 0.0031 VS 0.0215 ± 0.0044 g, $P = 0.06$, fig. 6G). The VEGFA-DSertG prostate showed less branching and a less differentiated morphology than the control (fig. 6A-F).

Effect of Sertoli cell-specific (sub-populations of germ cells) VEGFA loss on fertility:

To determine whether Sertoli cell-specific VEGFA loss has any effect on fertility, VEGFA-DSertG mice were mated with females that had VEGFA knocked out in granulosa and some germ cells (VEGFA-DGranG). There were 5 KO groups and 9 control groups. We found that the number of days for VEGFA-DGranG females mating

with VEGFA-DSertG males until parturition was longer than that for control females mating with control males (38.27 ± 4.65 VS 26.18 ± 2.17 days, $P < 0.05$, fig. 7).

Discussion:

In the present study, we used Cre-lox technology to knockout VEGFA in Sertoli cells and in sub-populations of germ cells. Our goal was to determine how this affected morphology and function. We demonstrated that removal of VEGFA in VEGFA-DSertG males affected growth since body weight, testis and epididymal weights were smaller with tendencies for the prostate to be smaller. Furthermore, *Bcl-2* mRNA expression also increased in VEGFA-DSertG testis, suggesting that increased cell apoptosis may occur. Furthermore, the Sertoli cell-specific VEGFA loss affected the morphology of epididymis and prostate resulting in fewer tubules in the epididymis and reduced branching in the prostate.

VEGFA is a key regulator of endothelial growth and permeability. However, an increasing number of reports suggest that VEGFA has direct effects on nonvascular cells including granulosa cells (Greenaway *et al.* 2004) and germ cells (Caires *et al.*, 2009). For transgenic mice, losing one of the VEGFA alleles also results in embryonic death before testis development in utero (Ferrara *et al.* 1996). But the role of VEGFA in extravascular cells has not yet been understood. We are the first to generate the Sertoli cell-specific VEGFA loss mice. Because the VEGFA was conditional knockout, the embryonic lethality did not occur. The adult mice were used to observe the effect of VEGFA on testis morphology and function.

Since previous studies in our laboratory demonstrated that rat testes treated with inhibitors of VEGFA receptors and/or antagonists to VEGFA ligands during sex differentiation had decreased vessel density (Bott *et al.*, 2006); we initially proposed that elimination of VEGFA in Sertoli cells may affect formation of the testis. This is supported by other reports of VEGFA angiogenic isoform overexpression in testis also increased capillary density in mice (Korpelainen *et al.*, 1998). These data suggest that VEGFA plays an important role in testis development. It may regulate the testis growth through regulating vessel density and thus the smaller testis in VEGFA-DSertG may due to less vessel density and inability of the testis to grow and develop due to inhibition of proliferation and migration of necessary cells from the mesonephros. Our laboratory has demonstrated that mesonephric cells expressing KDR appear to migrate into the developing testis and surround developing seminiferous cords at E11.5 in the mouse. Inhibition of VEGFA signal transduction impaired the migration of endothelial cells in cultured gonad. Thus, VEGFA may regulate the endothelial cell migration in gonads (Bott *et al.*, 2010).

Secondly, we proposed that the reduced size of testis in the VEGFA-DSertG KO mice was due to reduced total numbers of germ cells and developing sperm that were present within the testis. There is supporting evidence that KDR is expressed in germ cells during embryonic period and after birth in the testes suggesting that VEGFA may affect differentiation and proliferation of spermatogonia (Bott *et al.*, 2010). In bovine testis, VEGFA164 treatment caused more germ cells (Caires *et al.*, 2009). The VEGFA overexpression in testis induced spermatogenic arrest and infertility in mice (Korpelainen

et al., 1998). However, when we used VASA staining to determine germ cells numbers there was no differences in VEGFA-DSertG males and controls on the randomly counted areas of the testis. There does not appear to be any direct effect on germ cell numbers on randomly counted areas of the testis. But the total number of germ cell may be altered, so overall size of the testis was affected.

In the present study, the expression of *Bcl-2* and *Bcl-2/Bax* were increased in VEGFA-DSertG testis. One possibility is due to increased germ cell apoptosis signaling that mice need more *Bcl-2* to prevent the apoptosis. Recombinant mouse VEGFA₁₆₄ treatment increased numbers of germ cells in bovine testes following 5 days of culture when compared with controls. Increased expression of *Bcl-2* relative to *Bax* and decreased expression of *Bnip3* indicated VEGFA₁₆₄ also stimulated an intracellular response that prevents germ cell death in bovine testis tissue explants. Blocking VEGFA activity *in vitro* reduced the number of germ cells in VEGFA-treated testis tissue (Caires *et al.*, 2009). This suggests that VEGFA angiogenic isoform is a survival factor in germ cell. Loss of all VEGFA isoforms may result in less germ cells developing and reduced spermatogenesis. Increased germ cell apoptosis signaling may stimulate expression of *Bcl-2* to support germ cell survival and sperm production. Increased amount of *Bcl-2* may also be compensating for reduced VEGFA angiogenic isoforms. Besides, the higher *Bcl-2* mRNA and *Bcl-2/Bax* in KOs, the *Sin3a* mRNA also increased in VEGFA-DSertG testes. It is possible like *Bcl-2*, increased germ cell apoptosis or reduced sperm numbers stimulate more *Sin3a* to support spermatogenesis.

Testosterone concentration in plasma tended to be higher in VEGFA-DSertG mice than controls because three VEGF-DSertG males had 10 fold greater testosterone concentrations than controls (12.81 vs 0.97 ng/ml). One *in vitro* study showed that VEGF angiogenic isoforms increased testosterone production in mouse TM3 Leydig cells by a dose-dependent manner. Administration of anti-VEGF antibody to block the signaling through VEGF receptor resulted in an inhibitory effect of testosterone secretion (Hwang *et al.*, 2007). However, an ectopic study showed no relationship between VEGF angiogenic isoform treatment and serum testosterone concentration. In that study, bovine testis tissue was treated with VEGFA₁₆₄ before grafting onto castrated immunodeficient nude mice. Serum testosterone concentrations in mice with cultured testis tissue grafts treated with VEGFA₁₆₄ were not different compared to mice receiving control treatment (Schmidt *et al.*, 2006). The role of VEGF in production of testosterone is still unclear. In the current study, testosterone levels in three VEGFA-DSertG mice were 10 fold higher compared to controls. In VEGFA-DSertG mice, both VEGF angiogenic and inhibitory isoforms were knocked out in Sertoli cells and some germ cells. Loss of VEGF in Sertoli cells may affect the Leydig cell function and then cause the increase in serum testosterone.

In testis, LH secreted from anterior pituitary through cAMP, stimulates synthesis and release of testosterone by the Leydig cells. Aromatase converts testosterone to estrogen. We did aromatase immunohistochemistry in the testis. Some studies reported that estrogen is synthesis in Leydig cells and germ cell in the mouse (Wang *et al.*, 2001; Bilinska *et al.*, 2003; Carreau *et al.*, 2003; Hess and Carnes, 2004). In our studies,

aromatase located in Leydig cells in the interstitial area, some germ cells and Sertoli cells. VEGFA-DSertG testis aromatase did not present a difference compared to the controls. QPCR showed that *aromatase* mRNA in VEGFA-DSertG testis was not different with that in control. It is possible since we were only knocking out VEGFA in Sertoli cells that we were unable to detect differences in genes that are not specific to Sertoli cells. The result indicated that in VEGFA-DSertG mice, the VEGFA loss influenced the testosterone production via other enzyme or pathways potentially not through aromatase.

Body growth and male accessory gland organ development are affected by the testosterone. The increase in testosterone synthesis in male body after birth leads to a male pattern of growth hormone secretion and affects body growth. The testosterone level might be deficient at certain stages during development in VEGFA-DSertG mice. Thus, deficient testosterone may cause the lower weight of the body, testes and epididymis in VEGFA-DSertG mice. The VEGFA-DSertG prostate weight also tended to be smaller.

The epididymis is derived from the Wolffian duct and consists mainly of mesenchymal tissue at birth (Cornwall, 2009). Androgens are important for normal epididymal morphology and function while LH could be required for certain facets of epididymal morphology and function (Lei *et al.*, 2003). In present study, the high testosterone in VEGFA-DSertG could reduce the release of LH. Deficient LH may affect the epididymal growth. Another possibility for smaller epididymis is that the testosterone level might be deficient at stages during epididymal development.

Besides androgens, the development of differentiated epithelium also requires the influence of luminal factors from the testis (Rodriguez *et al.*, 2002) which are poorly understood. Ductus epididymidis was dilated in the VEGFA overexpressing transgenic mice. The number of subepithelial capillaries in the epididymis was increased in transgenic mice. This indicated that VEGFA is involved in the vessel development in epididymis (Korpelainen *et al.*, 1998). In current study, Sertoli cell-specific VEGFA loss may affect epididymal growth through influencing vessel development and permeability which could reduce epididymal size.

The prostate is an androgen target organ whose growth, morphology, and function are dependent on the androgens produced by the testes. Five- α -dihydrotestosterone (DHT) stimulates the development and growth of the prostate, but is not essential for prostatic bud formation (Mahendroo *et al.*, 2001). Androgens are required for prostatic development through the androgen receptor (Wilson *et al.*, 1995) and mesenchyme drives prostatic epithelial development (Thomson *et al.*, 2008). Regional differences in branching morphogenesis and cytodifferentiation are controlled by the instructional influences of mesenchyme and temporal expression of growth factors (Timms *et al.*, 2008). Sertoli cell-specific VEGFA loss may affect the size of prostate through DHT which was not measured in the present study. Branching morphogenesis in VEGFA-D_{SertG} was abnormal, suggesting prostate development may be altered by hormones or reduced growth factors.

In the present study, the time period from mating to parturition in female VEGFA-D_{GranG} X VEGFA-D_{SertG} mice was longer compared to the control. This increase in

time to pregnancy may be caused by problems with the reduction in female ovulation or reduction in spermatogenesis or potentially reduced sperm cell viability due to less than optimal seminal fluid quality. VEGFA-DSertG mice may lose expression of VEGFA in some germ cells. VEGFA concentration in human semen is a factor that predicts pregnancy success following IVF. Thus, too high or too low VEGFA in human semen is also related to pregnancy failure (Huminiecki *et al.*, 2001) and may contribute to the longer time to conceive and have pups in our KO models.

Removal of VEGFA in Sertoli cells caused mice to be sub-fertile and resulted in smaller body size, testis and epididymis. The serum testosterone level also tended to be high in VEGFA-DSertG. In VEGFA-DSertG mice, the morphology of epididymis and prostate was also altered. Sertoli cell-specific VEGFA loss also increased the *Bcl-2* and *Sin3a* expression in testis. Therefore, VEGFA plays an important role in male reproductive organ morphogenesis and function for mice.

Figure Legends

Table 1. Quantitative Real-time PCR primers, probes and kit.

Figure 1. Effect of Sertoli cell-specific VEGFA loss on the weights of male body (G) and testes (H). Histological images of sections (H&E stained) of control testis (A, C, E) and VEGFA-DSertG testis (B, D, F). The mean weights \pm SEM are presented. * denotes significant difference at $P < 0.05$.

Figure 2. Expression of *Bcl-2* (A), *Bax* (B) and the ratio of *Bcl-2/Bax* (C) and *Sin3a* (D) mRNA in testis. Figure E is amount of *Sin3a* mRNA in VEGFA-DSertG males with high testosterone. The mean normalized values \pm SEM are presented. ** denotes significant difference at $0.05 < P < 0.1$. * denotes significant difference at $P < 0.05$.

Figure 3. Effect of Sertoli cell-specific VEGFA loss on serum testosterone concentration (G). Three VEGFA-DSertG males had 10 fold greater testosterone concentrations than controls (H). Aromatase IHC in control testis (A, C) and VEGFA-DSertG testis (B, D). Negative control in IHC for control testis (E) and VEGFA-DSertG testis (F). Expression of *Aromatase* mRNA in the testis (I). The mean serum concentrations \pm SEM are presented. The mean normalized values \pm SEM are presented. ** denotes significant difference at $0.05 < P < 0.1$.

Figure 4. VASA immunohistochemistry in testis. VASA (a known germ cell marker) IHC in control testis (A, C) and VEGFA-DSertG testis (B, D). Negative control in IHC for control testis (E) and VEGFA-DSertG testis (F).

Figure 5. Effect of Sertoli cell-specific VEGFA loss on weight of epididymis (G). Histological images of sections (H&E stained) of control epididymis (A, C, E) and VEGFA-DSertG epididymis (B, D, F). The mean weights \pm SEM are presented. * denotes significant difference at $P < 0.05$.

Figure 6. Effect of Sertoli cell-specific VEGFA loss on weight of prostate (G). Histological images of sections (H&E stained) of control prostate (A, C, E) and VEGFA-DSertG prostate (B, D, F). The mean weights \pm SEM are presented. ** denotes significant difference at $0.05 < P < 0.1$.

Figure 7. Time from mating control female with control male to parturition VS time from mating VEGFA-DGranG females with VEGFA-DSertG males to parturition. There were 5 KO groups and 9 control groups. The mean days from mating to parturition \pm SEM are presented. * denotes significant difference at $P < 0.05$.

Gene	Forward Primer	Probe Dye	Probe Vendor	Reverse Primer
<i>Gapdh</i>	Kit-Taqman Rodent GapDH Control Reagents Applied Biosystems Catalog #4308313 (VIC Probe)			
<i>Bcl-2</i>	CGTTGGCCCTTCGGAGT T	FAM	Applied Biosystems	TCAGACCTCCAGGGCAGAGA
<i>Bax</i>	GGCCTTTTTGCTACAGG GTTT	FAM	Applied Biosystems	GTGTCTCCCCAGCCATCCT
<i>Sin3a</i>	Kit-Taqman Gene Expression Assay Mm00488255_m1 (FAM Probe)			
<i>Aromatase</i>	AACCCGAGCCTTTGGAG A	FAM	Applied Biosystems	GGCCCGTCAGAGCTTTCA

Table 1.

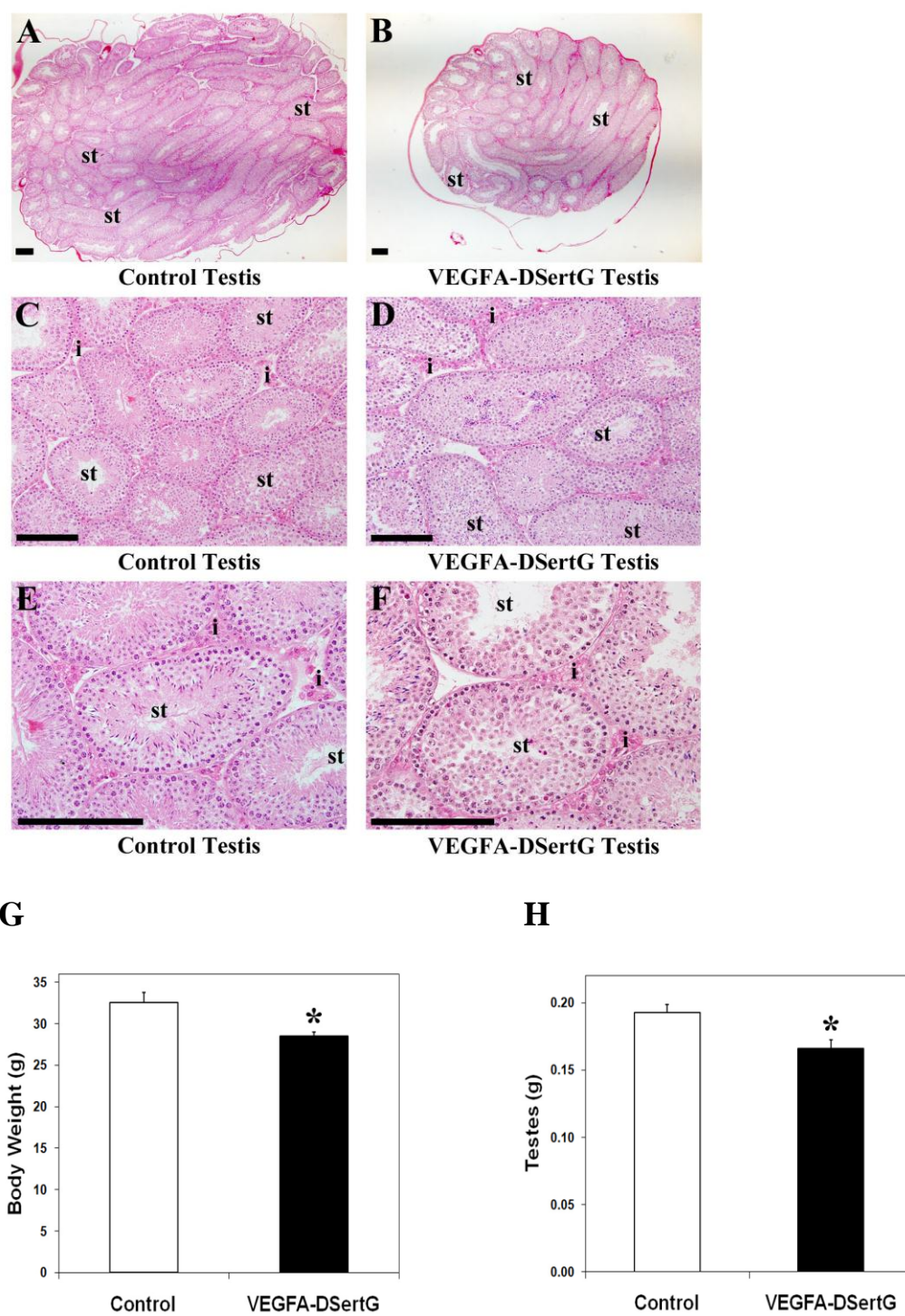
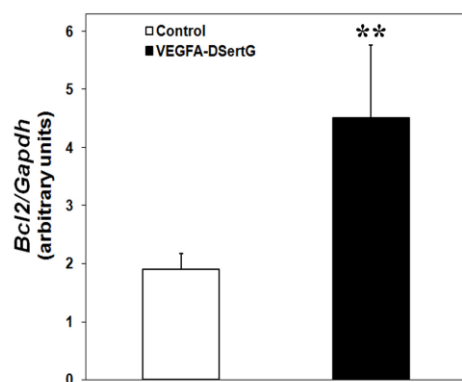
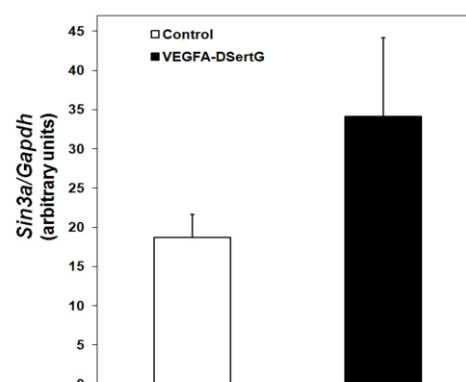
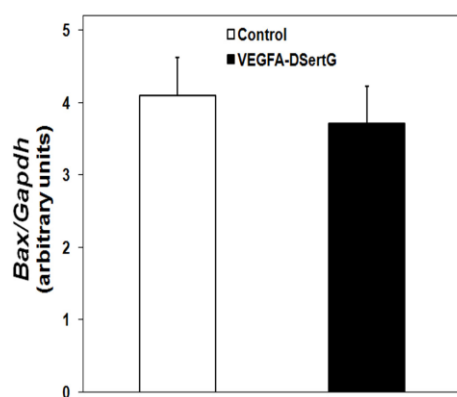
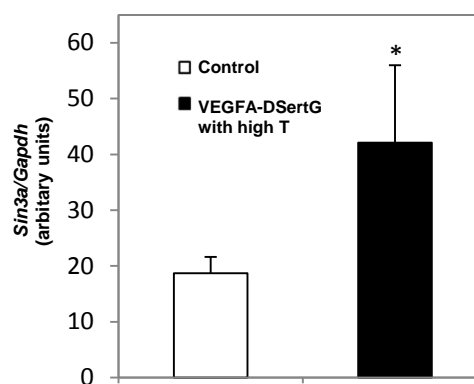
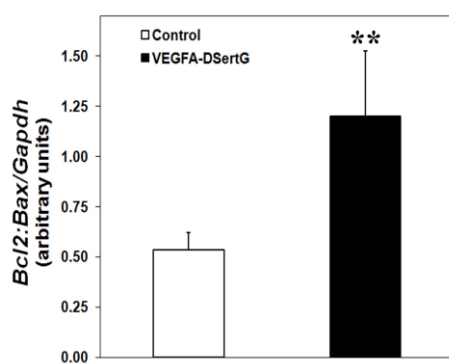
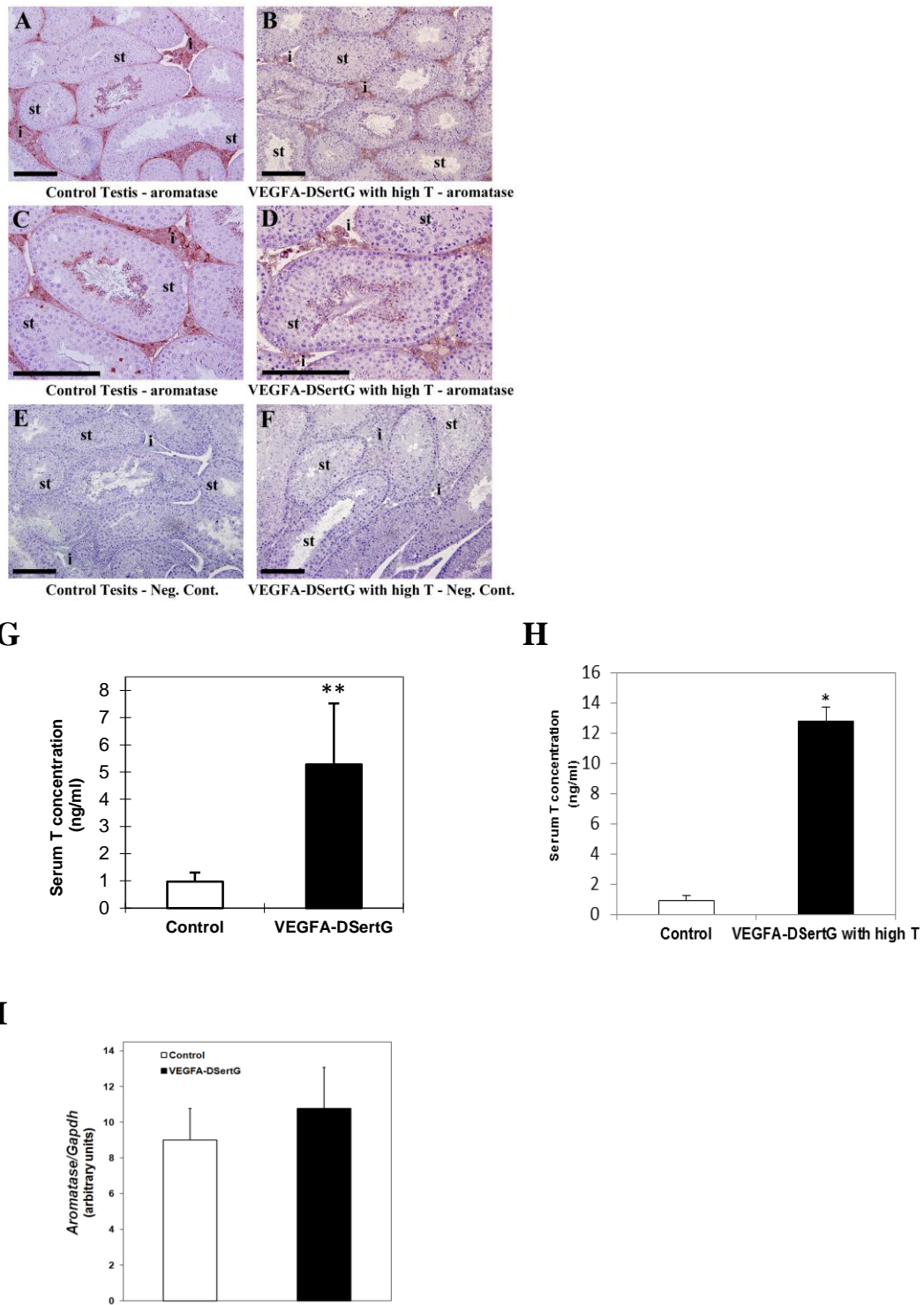


Figure 1.

A**D****B****E****C****Figure 2.**

**Figure 3.**

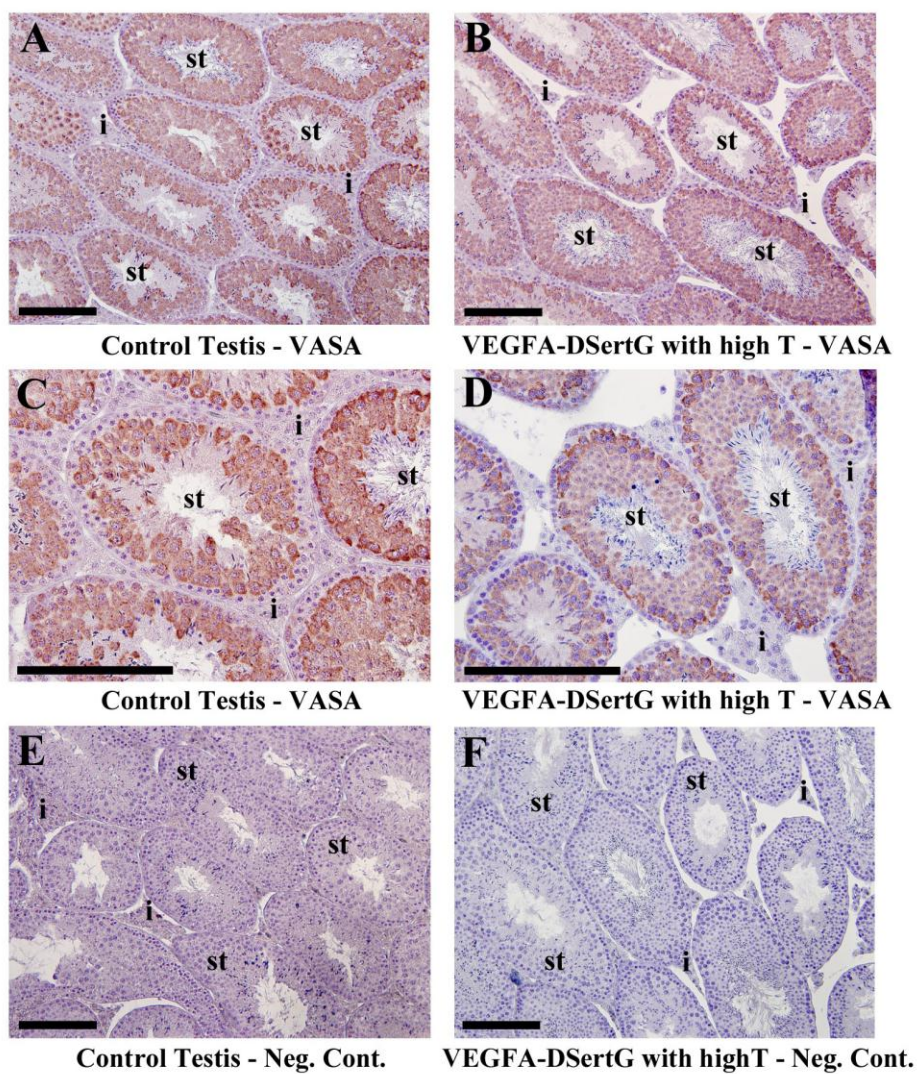


Figure 4.

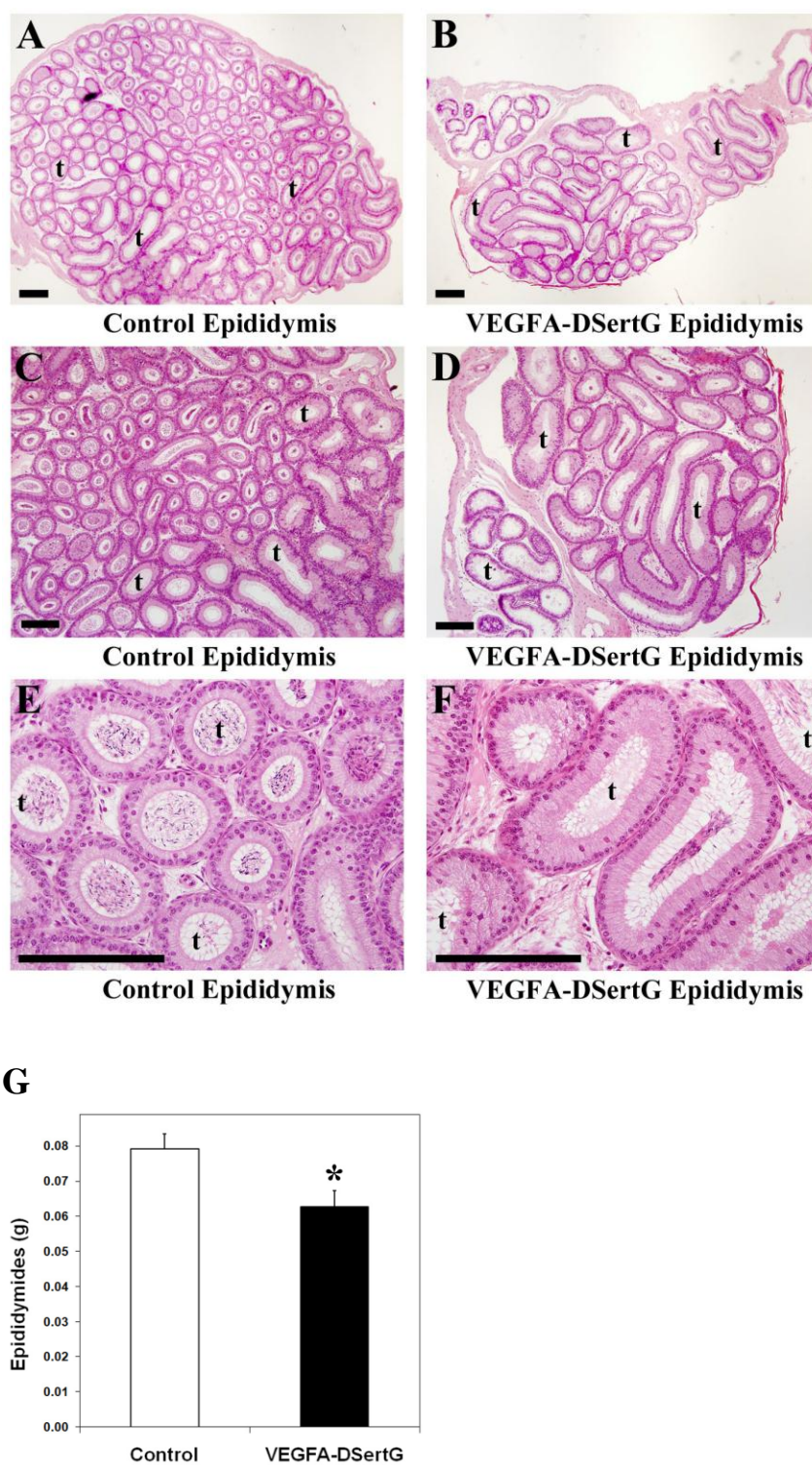


Figure 5.

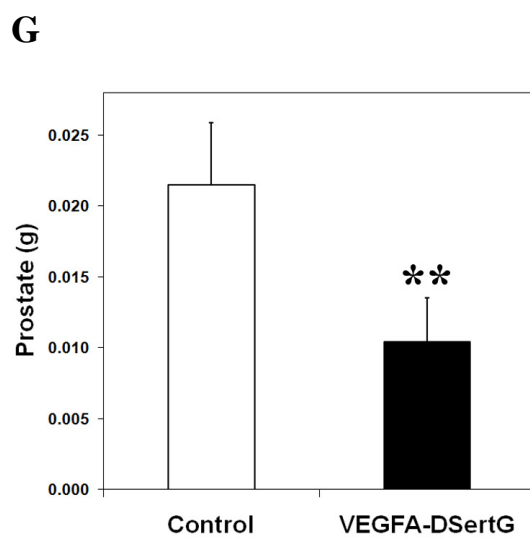
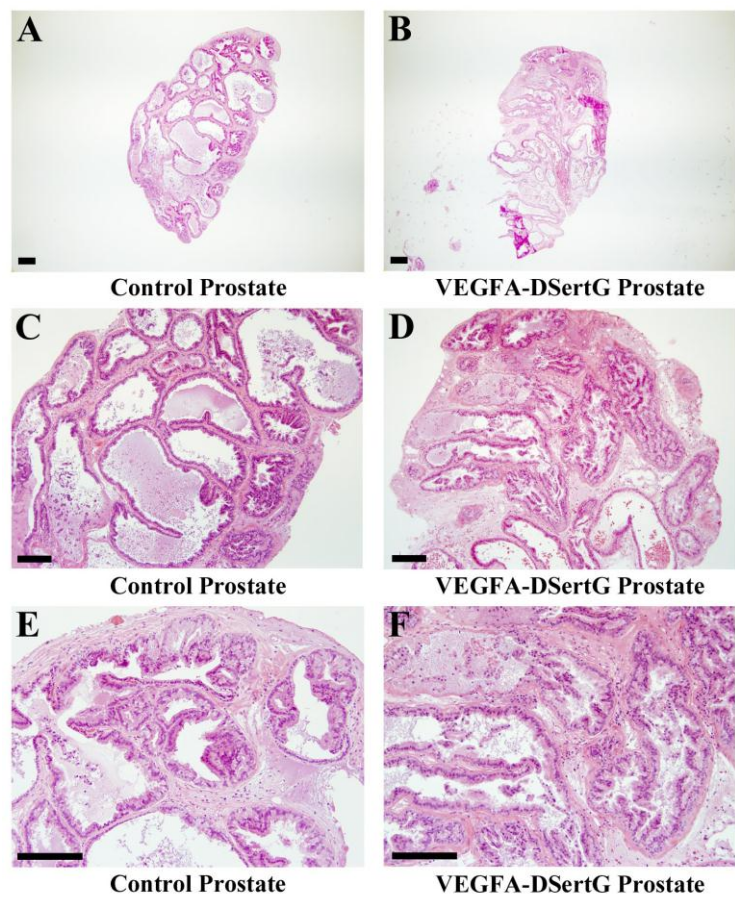


Figure 6.

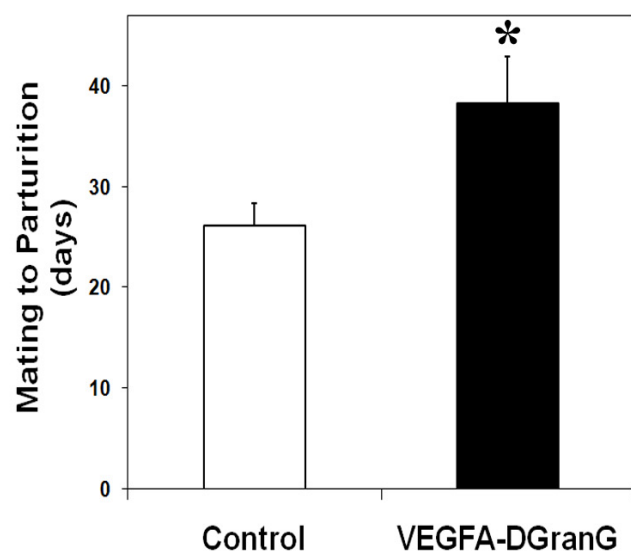


Figure 7.

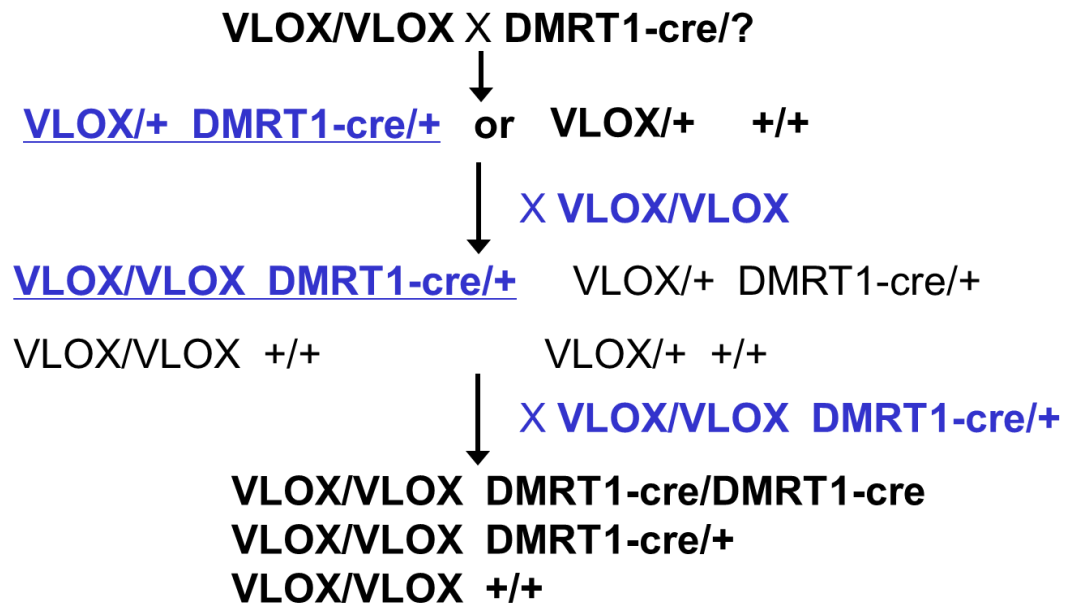
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Appendix A: VEGFA Conditional Knockout Breeding Scheme



Appendix B: Conventional PCR primers and programs for genotyping

Gene	Primer		PCR program		
<i>Dmrt-1</i>	Forward	5`-AGCAGAGGCTTCCTTCGACTT-3`	1	94 °C	4:00
			2	94 °	1:00
			3	60 °	0:15
	Reverse	5`-AGTGAACGAACCTGGTCGAAA-3`	4	72 °	0:15
			5	Go to 2	30 times
			6	2 °	5:00
			7	4 °	forever
<i>VEGFA^{lox/lox}</i>	Forward VEGFA419	5`-CCTGGCCCTCAAGTACACCTT-3`	1	50 °C	2:00
			2	95 °	10:00
			3	95 °	0:15
			4	57 °	1:00
	Reverse VEGFA567	5`- TCCGTACGACGCATTTCTAG-3`	5	72 °	2:00
			6	Go to 3	34 times
			7	72 °	3:00
			8	4 °	forever
<i>Sry</i>	Forward	5`-CGGGATCCATGTCAAGCGCCCATGA-3`	1	94 °C	1:00
			2	94 °	1:00
			3	58 °	1:00
	Reverse	5`- GCGGAATTCACCTTAGCCCTCCGATG-3`	4	72 °	1:00
			5	Go to 2	29 times
			6	72 °	5:00
			7	4 °	forever

Summary

In this thesis, we have demonstrated that the effects of Granulosa and Sertoli-cell specific VEGFA loss on gonads, male accessory gland, organ function and morphogenesis *in vivo*. We believe that the data in this thesis support our hypothesis that VEGFA is critical for normal gonadal morphogenesis and function.

We utilized Cre-lox technology to knockout VEGFA in female Granulosa cells, male Sertoli cells and sub-populations of germ in mice. We are the first to generate the female Granulosa cell (VEGFA-DGranG), and male Sertolic cell-specific (VEGFA-DSertG) VEGFA loss mice. In our conditional knockout mice both VEGFA pro-angiogenic and anti-angiogenic isoforms were removed.

We have identified that removal of VEGFA in Granulosa cells within ovaries resulted in smaller ovary, fewer CL, abnormal CL structure, and reduced 17beta-estradiol concentrations in blood plasma. Removal of VEGFA in VEGFA-DSertG males affected body weight, size of the testis, epididymis and prostate, as well as increasing testosterone concentrations (in three KO mice). In addition, *Bcl-2* mRNA expression also tended to be increased in VEGFA-DSertG testis when compared to controls and *Sin3a* mRNA was increased in those three VEGFA-DSertG males with high serum testosterone concentration than controls. Sertoli cell-specific VEGFA loss affected the morphology of epididymis and prostate with fewer tubules in the epididymus and reduced branching in the prostate. The time period from mating to parturition in female VEGFA-DGranG X male VEGFA-DSertG mice was longer compared to the control. This longer time to

conception may be due to reduced ovulation of the VEGFA-DGranG female mice and /or reduced quantity or quality of sperm in VEGFA-DSertG mice.

In summary, lack of VEGFA in Granulosa and Sertoli cells causes altered reproductive gonadal morphogenesis, reduced gonadal and male accessory gland organ weights, changed steroidogenic profiles and potentially caused subfertility in both male and female mice.